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(54) Title: **SUBTILISIN VARIANTS CAPABLE OF CLEAVING SUBSTRATES CONTAINING BASIC RESIDUES**

(57) Abstract

The bacterial serine protease, subtilisin BPN[®], has been mutated so that it will efficiently and selectively cleave substrates containing basic residues. Combination mutants, where Asn 62 was changed to Asp, Gly 166 was changed to Asp (N62D/G166D), and optionally Tyr 104 was changed to Asp had a larger than additive shift in specificity toward substrates containing basic residues. Suitable substrates of the subtilisin variants were revealed by sorting a library of phage particles (substrate phage) containing five contiguous randomized residues. This method identified a particularly good substrate, Asn-Leu-Met-Arg-Lys- (SEQ ID NO: 35), that was selectively cleaved in the context of a fusion protein by the N62D/G166D subtilisin variant. A particularly good substrate for N62D/G166D/Y104D would be Asn-Arg-Met-Arg-Lys- (SEQ ID NO: 76). Accordingly, these subtilisin variants are useful for cleaving fusion proteins with basic substrate linkers and processing hormones or other proteins *in vitro* or *in vivo* that contain basic cleavage sites.

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SUBTILISIN VARIANTS CAPABLE OF CLEAVING SUBSTRATES CONTAINING BASIC RESIDUES

FIELD OF THE INVENTION

This invention relates to subtilisin variants having altered specificity from wild-type subtilisins. Specifically, the subtilisin variants are modified so that they efficiently and selectively cleave substrates containing basic residues. The invention further relates to the DNA encoding these novel polypeptides, as well as the recombinant materials and methods for producing these subtilisin variants. In a particular aspect, the present invention provides for processes for cleaving protein substrates containing basic residues.

BACKGROUND OF THE INVENTION

Site-specific proteolysis is one of the most common forms of post-translational modifications of proteins (for review see Neurath, H. (1989) Trends Biochem. Sci., 14:268). In addition, proteolysis of fusion proteins *in vitro* is an important research and commercial tool (for reviews see Uhlen, M. and Moks, T. (1990) Methods Enzymol., 185:129-143; Carter, P. (1990) in Protein Purification: From Molecular Mechanisms to Large-Scale Processes, M.R. Landisch, R.C. Wilson, C.D. Painton, S.E. Builder, Eds. (ACS Symposium Series 427, American Chemical Society, Washington, D.C.), Chap. 13, p.181-193; and Nilsson, B. et al. (1992) Current Opin. Struct. Biol., 2:569). Expressing a protein of interest as a fusion protein facilitates purification when the fusion contains an affinity domain such as glutathione-S-transferase, Protein A or a poly-histidine tail. The fusion domain can also facilitate high level expression and/or secretion.

To liberate the protein product from the fusion domain requires selective and efficient cleavage of the fusion protein. Both chemical and enzymatic methods have been proposed (see references above). Enzymatic methods are generally preferred as they tend to be more specific and can be performed under mild conditions that avoid denaturation or unwanted chemical side-reactions. A number of natural and even designed enzymes have been applied for site-specific proteolysis. Although some are generally more useful than others (Forsberg, G., Baastrup, B., Rondahl, H., Holmgren, E., Pohl, G., Hartmanis, M. and Lake, M. (1992) J. Prot. Chem., 11:201-211), no one is applicable to every situation given the sequence requirements of the fusion protein junction and the possible existence of protease sequences within the desired protein product. Thus, an expanded array of sequence specific proteases, analogous to restriction endonucleases, would make site-specific proteolysis a more widely used method for processing fusion proteins or generating protein/peptide fragments either *in vitro* or *in vivo*.

The processing of prohormones by the KEX2-related family of serine endoproteases illustrates one of the most precise proteolytic events found in nature (for reviews see Steiner, D. F., Smeekens, S. P., Ohagi, S. and Chan, S. J. (1992) J. Biol. Chem., 267, 23435-23438 and Smeekens, S. P. (1993) Bio/Technology 11, 182-186). This family of proteases, that includes the yeast KEX2 and the mammalian PC2, PC3 and furin enzymes, are homologous to the bacterial serine protease subtilisin (Kraut, J. (1977) Annu. Rev. Biochem., 46:331-358). Subtilisin has a broad substrate specificity that reflects its role as a scavenger protease. In contrast, these eukaryotic enzymes are very specific for cleaving substrates containing two basic residues and thus well-suited for site-specific proteolysis.

All of these eucaryotic enzymes strongly require Arg at the P1 position, and either Arg, Lys or Pro at the P2 position of peptide substrates. The prohormone convertases from higher eukaryotes such as furin, PC2, and PC3 also have an absolute requirement for Arg at the P4 position (Bresnahan, P. A., Leduc, R., Thomas, L., Thomer, J., Gibson, H. L., Brake, A. J., Barr, P. J. and Thomas, G. (1990) *J. Cell. Biol.* 111, 2851; Wise, R. J., Baar, P. J., Wong, P. A., Kiefer, M. C., Brake, A. J., and Kaufman, R. J. (1990) *Proc. Natl. Acad. Sci. USA* 87, 9378-9382.; Hosaka, M., Nagahama, M., Kim, W.-S., Watanabe, T., Hatsuzakawa, K., Ikemizu, J., Murakami, K., and Nakayama, K. (1991) *J. Biol. Chem.* 266, 12127-12130.; Matthews, D. J., Goodman, L. J., Gorman, C. M., and Wells, J. A. (1994) *Protein Science* 3, 1197-1205).

Despite the very narrow specificity of the pro-hormone processing enzymes, in some cases they are capable of rapid cleavage of target sequences. For example, the k_{cat}/K_m ratio for KEX2 to cleave a good substrate (e.g. acetyl-pMYRK-MCA) is $1.1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (Brenner, C., and Fuller, R.S. (1992) *Proc. Natl. Acad. Sci. USA* , 89:922-926) compared to 3×10^5 for subtilisin cleaving a good substrate (e.g. suc-AAPF-pNA) (Estell, D. A., Graycar, T. P., Miller, J. V., Powers, D. B., Burnier, J. P., Ng, P. G. and Wells, J.A. (1986) *Science*, 233:659-663).

However, the eukaryotic proteases are expressed in small amounts (Bravo, D. B., Gleason, J. B., Sanchez, R. I., Roth, R. A., and Fuller, R. S. (1994) *J. Biol. Chem.*, 269:25830-25837 and Matthews, D. J., Goodman, L. J., Gorman, C. M., and Wells, J. A. (1994) *Protein Science* , 3:1197-1205) making them impractical to apply presently to processing of fusion proteins *in vitro*. Subtilisin BPN' however, can be expressed in large amounts (Wells, J.A., Ferrari, E., Henner, D.J., Estell, D.A. and Chen, E.Y. (1983) *Nucl. Acids Res.*, 11:7911-7929)

Extensive protein engineering studies of subtilisin, and especially subtilisin BPN', have identified several residues in the S1 and S2 active site of the enzyme where amino acid substitutions lead to large changes in substrate specificity (Wells, J. A., and Estell, D.A., (1988) *Trends Biochem. Sci.*, 13:291-297; Carter, P., et al., (1989) *PROTEINS:Structure, Function, and Genetics*, 6:240-248). X-ray crystal structures of subtilisin containing bound transition state analogues (Wright, C. S., Alden, R. A. and Kraut, J. (1969) *Nature* , 221:235-242; McPhalen, C.A. and James, N.G. (1988) *Biochemistry*, 27:6582-6598; Bode, W., Papamokos, E., Musil, D., Seemueller, U. and Fritz, M. (1986) *EMBO J.*, 5:813-818; and Bott, R., Utsch, M., Kossiakoff, A., Graycar, T., Katz, B. and Power, S. (1988) *J. Biol. Chem.*, 263:7895-7906) can be used to locate active site residues that are in close proximity to side chains at key positions in substrate peptides (Wells, J.A., (1987) *Proc. Natl. Acad. Sci. USA* 84:1219-1223). Consideration of electrostatic interactions between charged peptide substrates and subtilisin can be used to tailor the substrate binding cleft of the subtilisin BPN' to favor complementary charged substrates (Wells, J.A., et al., (1987) *Proc. Natl. Acad. Sci., USA*, 84:1219-1223). Previous work has shown that replacement of residues at position 156 and 166 in the S1 binding site of subtilisin BPN' with various charged residues leads to improved specificity for complementary charged substrates.

A substantial amount of protein engineering has been applied to the specificity determinants of the S4 subsite of subtilisin BPN' in efforts to alter specificity for P4 substrates (Eder, J., Rheinneck, M., and Fersht, A. R. (1993) *FEBS Lett* 335, 349-352; Rheinneck, M., Baker, G., Eder, J., and Fersht, A. R. (1993) *Biochemistry* 32, 1199-1203; Rheinneck, M., Eder, J., Pandey, P.S., and Fersht, A. R. (1994) *Biochemistry* 33,

221-225). However, the mutations introduced consisted entirely of hydrophobic substitutions, thus preserving the overall hydrophobic substrate preference in the site.

Previous attempts to introduce, remove or reverse charge specificity in enzyme active sites have been met with considerable difficulty. This has generally been attributed to a lack of stabilization of the introduced charge or enzyme-substrate ion pair complex by the wild-type enzyme environment (Hwang, J.K. and Warshel, A. (1988) *Nature*, 334:270-272). For example, Stennicke *et al.* (Stennicke, H.R.; Ujje, H.M.; Christensen, U.; Remington, S.J.; and Breddam (1994) *Prot. Eng.* 7:911-916) made acidic (D/E) mutations at five residues in the P1' binding of carboxypeptidase Y in an attempt to change the P1' preference from Phe to Lys/Arg. Only the L272D and L272E mutations were found to alter the specificity in the desired direction, up to 1.5-fold preference in Lys/Arg over Phe, and the others simply resulted in less active enzymes having substrate preferences similar to wild-type. In the case of trypsin, a protease that is highly specific for basic P1 residues, recruitment of chymotrypsin-like (hydrophobic P1) specificity required not only mutations of the ion pair-forming Asp 189 to Ser, but also transplantation of two more distant surface loops from chymotrypsin (Graf, L., Jancso, A., Szilagyi, L., Hegyi, G., Pinter, K., Naray-Szabo, G., Hepp, J., Medzihradsky, K., and Rutter, W. J., *Proc. Natl. Acad. Sci. USA* (1988) 85:4961-4965 and Hedstrom, L., Szilagyi, L., and Rutter, W. J., *Science* (1992) 255:1249-1253).

In the present work, we have also verified that relatively low specificity is gained by introducing single ion-pairs between enzyme and substrate. However, when two or more choice ionic interactions were simultaneously engineered into subtilisin BPN', the resulting variants had higher specificity for basic residues in each of the subsites due to a non additive effect.

Accordingly, it is an object to produce a subtilisin variant with basic specificity for use in processing pro-proteins made by recombinant techniques.

SUMMARY OF THE INVENTION

The present invention provides for subtilisin variants with altered substrate specificity. Preferred subtilisin variants are highly specific for the efficient cleavage of substrates containing basic residues. The subtilisin variants have a substrate specificity which is substantially different from the substrate specificity of the precursor subtilisin from which the amino acid sequence of the variant is derived. The amino acid sequence of the subtilisin variants are derived by the substitution of one or more amino acids of a precursor subtilisin amino acid sequence.

In a preferred aspect of the present invention, the subtilisin variants of the present invention are specific for the cleavage of protein substrates containing basic amino acid residues at substrate positions P1, P2 and P4. According to this aspect of the present invention subtilisin variants having amino acid substitutions at positions corresponding to amino acid positions 62, 104 and 166 of subtilisin BPN' produced by *Bacillus amyloliquefaciens* are preferred. Accordingly, subtilisin variants are provided wherein amino acids 62, 104 and 166 of subtilisin BPN' are substituted with an acidic amino acids. Preferably the acidic amino acid is Asp or Glu. and most preferably Asp.

Preferred substrates for the subtilisin variants according to this aspect of the present invention contain either Lys (K) or Arg (R) at substrate positions P2 and P1, practically any residue at P3, and preferably either Lys or Arg at P4, and again practically any residue at P5. Thus an exemplary good substrate would contain -Asn-Arg-Met-Arg-Lys- (SEQ ID NO: 76) at -P5-P4-P3-P2-P1- respectively. Additionally, good substrates would not have Pro at P1', P2', or P3' nor would Ile be present at P1'.

According to a second aspect of the present invention the subtilisin variants are capable of cleaving protein substrates having basic residues at positions P1 and P2. According to this aspect of the present invention subtilisin variants having amino acid substitutions at positions corresponding to amino acid positions 62, and 166 of subtilisin BPN⁺ produced by *Bacillus amyloliquefaciens* are preferred. The preferred subtilisin variants having substrate specificity for dibasic substrates have an acidic amino acid residue at residue position 62 of subtilisin naturally produced by *Bacillus amyloliquefaciens*. In a preferred embodiment, the naturally occurring Asn at residue position 62 of subtilisin BPN⁺ is preferably substituted with an acidic amino acid residue such as Glu or Asp, and most preferably Asp. The preferred subtilisin variants, having substrate specificity for substrates having dibasic amino acid residues, additionally have an acidic residue, Asp or Glu, at residue position 166 of subtilisin BPN⁺. Thus, the subtilisin BPN⁺ variant containing substitution of amino acids 62 and 166 with acidic amino acids Glu or Asp are preferred. In particular, a subtilisin variant having amino acid Asp at positions 62 and 166 is preferred (subtilisin BPN⁺ variant N62D/G166D). The subtilisin variants according to this aspect of the invention may be used to cleave substrates containing dibasic residues such as fusion proteins with dibasic substrate linkers and processing hormones or other proteins (*in vitro* or *in vivo*) that contain dibasic cleavage sites.

Preferred substrates for the subtilisin BPN⁺ variant N62D/G166D contain either Lys (K) or Arg (R) at substrate positions P2 and P1, practically any residue at P3, a non-charged hydrophobic residue at P4, and again practically any residue at P5. Thus an exemplary good substrate would contain -Asn-Leu-Met-Arg-Lys- (SEQ ID NO: 35) at -P5-P4-P3-P2-P1- respectively. Additionally, good substrates would not have Pro at P1', P2', or P3' nor would Ile be present at P1'.

The invention also includes mutant DNA sequences encoding such subtilisin variants. These mutant DNA sequences are derived from a precursor DNA sequence which encodes a naturally occurring or recombinant precursor subtilisin. The mutant DNA sequence is derived by modifying the precursor DNA sequence to encode the substitution(s) of one or more amino acids encoded by the precursor DNA sequence. These recombinant DNA sequences encode mutants having an amino acid sequence which does not exist in nature and a substrate specificity which is substantially different from the substrate specificity of the precursor subtilisin encoded by the precursor DNA sequence.

Further the invention includes expression vectors containing such mutant DNA sequences as well as host cells transformed with such vectors which are capable of expressing the subtilisin variants.

The invention also provides for a process for cleaving a polypeptide such as a fusion protein containing a substrate linker represented by the formula:



wherein P4 is a basic amino acid or a large hydrophobic amino acid such as Leu or Met; P3 is an amino acid selected from the naturally occurring amino acids; P2 is a basic amino acid; and P1 is a basic amino acid. The

process includes the step of subjecting the polypeptide to the subtilisin variants described herein under conditions such that the subtilisin variant cleaves the polypeptide.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Structure of a succinyl-Ala-Ala-Pro-BoroPhe (SEQ ID NO: 69) inhibitor bound to the active site of subtilisin BPN' showing the S2 and S1 binding pocket residues subjected to mutagenesis.

Figure 2. Kinetic analysis of S1 binding site subtilisin mutants versus substrates having variable P1 residues. The kinetic constant k_{cat}/K_m was determined from plots of initial rates versus substrate concentration for the tetrapeptide series succinyl-Ala-Ala-Pro-Xaa-pNa (SEQ ID NO: 69), where Xaa was Lys (SEQ ID NO: 58), Arg (SEQ ID NO: 59), Phe (SEQ ID NO: 56), Met (SEQ ID NO: 60) or Gln (SEQ ID NO: 61) (defined to the right of the plot).

Figure 3. Kinetic analysis of S2 binding site subtilisin mutants versus substrates having variable P2 residues. The kinetic constant k_{cat}/K_m was determined from plots of initial rates versus substrate concentration for the tetrapeptide series succinyl-Ala-Ala-Xaa-Phe-pNa (SEQ ID NO: 70), where Xaa was Lys (SEQ ID NO: 62), Arg (SEQ ID NO: 64), Ala (SEQ ID NO: 63), Pro (SEQ ID NO: 56), or Asp (SEQ ID NO: 65) (defined on the right of the plot).

Figure 4. Kinetic analysis of combined S1 and S2 binding site subtilisin mutants versus substrates having variable P1 and P2 residues. The kinetic constants k_{cat}/K_m were determined from plots of initial rates versus substrate concentration for the tetrapeptide series succinyl-Ala-Ala-Xaa₂-Xaa₁-pNa (SEQ ID NO: 71), where Xaa₂-Xaa₁ was Lys-Lys (SEQ ID NO: 66), Lys-Arg (SEQ ID NO: 67), Lys-Phe (SEQ ID NO: 62), Pro-Lys (SEQ ID NO: 58), Pro-Phe (SEQ ID NO: 56), or Ala-Phe (SEQ ID NO: 63) (defined on the right of the plot).

Figure 5. Results of hGH-AP fusion protein assay. hGH-AP fusion proteins were constructed, bound to hGHbp-coupled resin, and treated with 0.5 nM N62D/G166D subtilisin in 20 mM Tris-Cl pH 8.2. Aliquots were withdrawn at various times and AP release was monitored by activity assay in comparison to a standard curve. Arrows indicate the cleavage site. The rate of cleavage of fusion proteins containing various substrate linkers is shown. Substrates containing a Pro at position P1' are not cleaved.

Figure 6-1 - 6-10. (Collectively referred to herein as Fig. 6). DNA sequence of the phagemid pSS5 containing the N62D/G166D double mutant subtilisin BPN' gene (SEQ ID NO: 1), and translated amino acid sequence for the mutant preprosubtilisin (SEQ ID NO: 2). The pre region is comprised of residues -107 to -78, the pro of residues -77 to -1, and the mature enzyme of residues +1 to +275 (SEQ ID NO: 72). Also shown are restriction sites recognized by endonucleases that require 6 or more specific bases in succession.

Figure 7. Structure of a succinyl-Ala-Ala-Pro-BoroPhe (SEQ ID NO: 69) inhibitor bound to the active site of subtilisin BPN' showing the S1, S2, and S4 binding pocket residues subjected to mutagenesis.

Figure 8. DNA sequence of the N62D/Y104D/G166D triple mutant (SEQ ID NO: 74) as well as the translated amino acid sequence (SEQ ID NO: 75). The preregion is comprised of residues -107 to -78, the pro residues -77 to -1 and the mature enzyme +1 to +275. The proregion reflects the changes, A(-4)R/A(-2)K/Y(-1)R made in the wild-type processing site to affect expression.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Terms used in the claims and specification are defined as set forth below unless otherwise specified.

The term amino acid or amino acid residue, as used herein, refers to naturally occurring L amino acids or residues, unless otherwise specifically indicated. The commonly used one- and three-letter abbreviations for amino acids are use herein (Lehninger, A. L., *Biochemistry*, 2d ed., pp. 71-92, Worth Publishers, N. Y. (1975)). Basic amino acids are Arg and Lys. Acidic amino acids are Asp and Glu.

Substrates are described in triplet or single letter code as Pn...P2-P1-P1'-P2'...Pn'. The "P1" residue refers to the position proceeding (i.e., N-terminal to) the scissile peptide bond (i.e. between the P1 and P1' residues) of the substrate as defined by Schechter and Berger (Schechter, I. and Berger, A., *Biochem. Biophys. Res. Commun.* 27: 157-162 (1967)). Similarly, the term P1' is used to refer to the position following (i.e., C-terminal to) the scissile peptide bond of the substrate. Increasing numbers refer to the next consecutive position preceding (e.g., P2 and P3) and following (e.g., P2' and P3') the scissile bond. According to the present invention the scissile peptide bond is that bond that is cleaved by the subtilisin variants of the instant invention.

"Subtilisins," "precursor subtilisin" and the like are bacterial carbonyl hydrolases which generally act to cleave peptide bonds of proteins or peptides. As used herein, "subtilisin" means a naturally occurring subtilisin or a recombinant subtilisin. A series of naturally occurring subtilisins are known to be produced and often secreted by various bacterial species (Siezen, R.J., et al., (1991) *Protein Engineering* 4:719-737). Amino acid sequences of the members of this series are not entirely homologous. However, the subtilisins in this series exhibit the same or similar type of proteolytic activity. This class of serine proteases shares a common amino acid sequence defining a catalytic triad which distinguishes them from the chymotrypsin related class of serine proteases. The subtilisins and chymotrypsin related serine proteases both have a catalytic triad comprising aspartate, histidine and serine. In the subtilisin related proteases the relative order of these amino acids, reading from the amino to carboxy terminus is aspartate-histidine-serine. In the chymotrypsin related proteases the relative order, however is histidine-aspartate-serine. Thus, subtilisins as used herein refer to a serine protease having the catalytic triad of subtilisin related proteases.

Generally, subtilisins are serine endoproteases having molecular weights of about 27,500 which are secreted in large amounts from a wide variety of *Bacillus* species. The protein sequence of subtilisins have been determined from at least four different species of *Bacillus* (Markland, F.S., et al. (1971) in *The Enzymes*, ed. Boyer P.D., Acad Press, New York, Vol. III, pp. 561-608; and Nedkov, P. et al. (1983) Hoppe-Seyler's Z. Physiol. Chem. 364:1537-1540). The three-dimensional crystallographic structure of four subtilisins have been reported (BPN' from *Bacillus amyloliquefaciens*, Hirono et al. (1984) *J. Mol. Biol.* 178:389-413; subtilisin Carlsberg from *Bacillus licheniformis*, Bode et al., (1986) *EMBO J.* 5:813-818; thermitase from *Thermoactinomyces vulgaris*, Gros et al., (1989) *J. Mol. Biol.* 210:347-367; and proteinase K from *Tritirachium album*, Betzel, et al., (1988) *Acta Crystallogr., B*, 44:163-172). The three dimensional structure of subtilisin BPN' (from *B. amyloliquefaciens*) to 2.5Å resolution has also been reported by Wright, C.S. et al. (1969) *Nature* 221:235-242 and Drenth, J. et al. (1972) *Eur. J. Biochem.* 26:177-181. These studies indicate that although subtilisin is genetically unrelated to the mammalian serine proteases, it has a similar fold and active site structure. The x-ray crystal structures of subtilisin containing covalently bound peptide inhibitors (Robertus, J.D., et al.

(1972) *Biochemistry* 11:2439-2449), product complexes (Robertus, J.D., et al. (1972) *Biochemistry* 11:4293-4303), and transition state analogs (Matthews, D.A., et al. (1975) *J. Biol. Chem.* 250:7120-7126 and Poulos, T.L., et al. (1976) *J. Biol. Chem.* 251:1097-1103), which have been reported have also provided information regarding the active site and putative substrate binding cleft of subtilisins. In addition, a large number of kinetic and chemical modification studies have been reported for subtilisins (Phillip, M., et al. (1983) *Mol. Cell. Biochem.* 51:5-32; Svendsen, I.B. (1976) *Carlsberg Res. Comm.* 41:237-291 and Markland, F.S. *Id.*) as well as at least one report wherein the side chain of methione at residue 222 of subtilisin was converted by hydrogen peroxide to methionine-sulfoxide (Stauffer, D.C., et al. (1965) *J. Biol. Chem.* 244 5333-5338).

"Subtilisin variant," "subtilisin mutant" and the like refer to a subtilisin-type serine protease having a sequence which is not found in nature that is derived from a precursor subtilisin according to the present invention. The subtilisin variant has a substrate specificity different from the precursor subtilisin by virtue of amino acid substitutions within the precursor subtilisin amino acid sequence. The term is meant to include subtilisin variants in which the DNA sequence encoding the precursor subtilisin is modified to produce a mutant DNA sequence which encodes the substitution of one or more amino acids in the naturally occurring subtilisin amino acid sequence. Suitable methods to produce such modification include those disclosed in U. S. Patent No. 4,760,025 and 5,371,008 and in EPO Publication No. 0130756 and 0251446.

A change in substrate specificity is defined as a difference between the K_{cat}/K_m ratio of the precursor subtilisin and the subtilisin variant. The K_{cat}/K_m ratio is a measure of catalytic efficiency. Subtilisin variants with increased or decreased K_{cat}/K_m ratios compared to the precursor subtilisin from which they were derived are described herein. Generally, the objective is to secure a variant having a greater, i.e. numerically larger, K_{cat}/K_m ratio for a given substrate. A greater K_{cat}/K_m ratio for a particular substrate indicates that the variant may be used to more efficiently cleave the target substrate.

The specificity or discrimination between two or more competing substrates is determined by the ratios of k_{cat}/K_m (Fersht, A.R., (1985) in *Enzyme Structure and Mechanism*, W.F. Freeman and Co., N.Y. p. 112). An increase in K_{cat}/K_m ratio for one substrate may be accompanied by a reduction in K_{cat}/K_m ratio for another substrate. This shift in substrate specificity indicates that the variant subtilisin with the increased K_{cat}/K_m ratio for the substrate has utility in cleaving the particular substrate over the precursor subtilisin in, for example, preventing undesirable hydrolysis of a particular substrate in a mixture of substrates.

In general, for a subtilisin variant to have a useful catalytic efficiency for cleavage of a particular substrate the K_{cat}/K_m ratio will generally be between $1 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ to about $1 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$. More often, the K_{cat}/K_m ratio will be between about $1 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ and $1 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$.

When referring to mutants or variants, the wild type amino acid residue is followed by the residue number and the new or substituted amino acid residue. For example, substitution of D for wild type N in residue position 62 is denominated N62D.

"Subtilisin variants or mutants" are designated in the same manner by using the single letter amino acid code for the wild-type residue followed by its position and the single letter amino acid code of the replacement residue. Multiple mutants are indicated by component single mutants separated by slashes. Thus the subtilisin BPN' variant N62D/G166D is a di-substituted variant in which Asp replaces Asn and Gly at residue positions 62 and 166, respectively, in wild-type subtilisin BPN'.

An amino acid residue of a precursor carbonyl hydrolase is "equivalent" to a residue of *B. amyloliquefaciens* subtilisin if it is either homologous (i.e., corresponding in position in either primary or tertiary structure) or analogous to a specific residue or portion of that residue in *B. amyloliquefaciens* subtilisin (i.e., having the same or similar functional capacity to combine, react, or interact chemically).

5 In order to establish homology to primary structure, the amino acid sequence of a precursor carbonyl hydrolase is directly compared to the *B. amyloliquefaciens* subtilisin primary sequence and particularly to a set of residues known to be invariant in all subtilisins for which the sequences are known (see e.g. Figure 5-C in EPO 0251446). After aligning the conserved residues, allowing for necessary insertions and deletions in order to maintain alignment (i.e., avoiding the elimination of conserved residues through arbitrary deletion and insertion),
 10 the residues equivalent to particular amino acids in the primary sequence of *B. amyloliquefaciens* subtilisin are defined. Alignment of conserved residues should conserve 100% of such residues. However, alignment of greater than 75% or as little as 50% of conserved residues is also adequate to define equivalent residues. Conservation of the catalytic triad, Asp32/His64/Ser221, is required.

Equivalent residues homologous at the level of tertiary structure for a precursor carbonyl hydrolase whose
 15 tertiary structure has been determined by x-ray crystallography, are defined as those for which the atomic coordinates of 2 or more of the main chain atoms of a particular amino acid residue of the precursor carbonyl hydrolase and *B. amyloliquefaciens* subtilisin (N on N, CA on CA, C on C, and O on O) are within 0.13nm and preferably 0.1nm after alignment. Alignment is achieved after the best model has been oriented and positioned to give the maximum overlap of atomic coordinates of non-hydrogen protein atoms of the carbonyl hydrolase
 20 in question to the *B. amyloliquefaciens* subtilisin. The best model is the crystallographic model giving the lowest R factor for experimental diffraction data at the highest resolution available.

$$R \text{ factor} = \frac{\sum_h |F_o(h)| - |F_c(h)|}{\sum_h |F_o(h)|}$$

Equivalent amino acid residues of subtilisin BPN', subtilisin Carlsberg, thermitase and proteinase K from tertiary structure analysis is provided in, for example, Siezen, et al., (1991) Prot. Eng. 4:719-737.

30 Equivalent residues which are functionally analogous to a specific residue of *B. amyloliquefaciens* subtilisin are defined as those amino acids of the precursor carbonyl hydrolases which may adopt a conformation such that they either alter, modify or contribute to protein structure, substrate binding or catalysis in a manner defined and attributed to a specific residue of the *B. amyloliquefaciens* subtilisin as described herein. Further, they are those residues of the precursor carbonyl hydrolase (for which a tertiary structure has been obtained by
 35 x-ray crystallography), which occupy an analogous position to the extent that although the main chain atoms of the given residue may not satisfy the criteria of equivalence on the basis of occupying a homologous position, the atomic coordinates of at least two of the side chain atoms of the residue lie within 0.13nm of the

corresponding side chain atoms of *B. amyloliquefaciens* subtilisin. The three dimensional structures would be aligned as outlined above.

Some of the residues identified for substitution are conserved residues whereas others are not. In the case of residues which are not conserved, the replacement of one or more amino acids is limited to substitutions which produce a mutant which has an amino acid sequence that does not correspond to one found in nature. In the case of conserved residues, such replacements should not result in a naturally occurring sequence. The subtilisin mutants of the present invention include the mature forms of subtilisin mutants as well as the pro- and prepro-forms of such subtilisin mutants. The prepro-forms are the preferred construction since this facilitates the expression, secretion and maturation of the subtilisin mutants.

5 "Prosequence" refers to a sequence of amino acids bound to the N-terminal portion of the mature form of a subtilisin which when removed results in the appearance of the "mature" form of the subtilisin. Many proteolytic enzymes are found in nature as translational proenzyme products and, in the absence of post-translational processing, are expressed in this fashion. The preferred prosequence for producing subtilisin mutants, specifically subtilisin BPN' mutants, is the putative prosequence of *B. amyloliquefaciens* subtilisin although other subtilisin prosequences may be used. For example, when the substrate specificity of the precursor subtilisin is altered according to the present invention, this alteration may affect the ability of the variant subtilisin to undergo autolytic cleavage of the naturally occurring prosequence. In order to affect the expression and proper folding of a mature variant subtilisin whose substrate specificity has been altered, it may be necessary to alter the prosequence to correspond to the new or variant substrate specificity.

10 As an example, the substrate specificity of a particular subtilisin variant N62D/Y104D/G166D is distinct from the precursor subtilisin from which it was derived. The subtilisin variant prefers substrates containing basic residues at substrate positions corresponding to P4, P2, and P1. According to this aspect of the present invention, the precursor prosequence which was efficiently autolysed by the precursor subtilisin is altered to correspond to the substrate specificity of the variant subtilisin. Therefore, for the subtilisin variant N62D/Y104D/G166D the prosequence would be altered to contain basic residues at positions -4, -2, and -1.

15 A "signal sequence" or "presequence" refers to any sequence of amino acids bound to the N-terminal portion of a subtilisin or to the N-terminal portion of a prosubtilisin which may participate in the secretion of the mature or pro forms of the subtilisin. This definition of signal sequence is a functional one, meant to include all those amino acid sequences, encoded by the N-terminal portion of the subtilisin gene or other secretory carbonyl hydrolases, which participate in the effectuation of the secretion of subtilisin or other carbonyl hydrolases under native conditions. The present invention utilizes such sequences to effect the secretion of the subtilisin mutants as defined herein.

20 A "prepro" form of a subtilisin mutant consists of the mature form of the subtilisin having a prosequence operably linked to the amino-terminus of the subtilisin and a "pre" or "signal" sequence operably linked to the amino terminus of the prosequence.

25 "Expression vector" refers to a DNA construct containing a DNA sequence which is operably linked to a suitable control sequence capable of effecting the expression of the DNA in a suitable host. Such control sequences include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome binding sites, and sequences which control termination of

transcription and translation. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome. or may, in some instances, integrate into the genome itself. In the present specification, "plasmid" and "vector" are sometimes used interchangeably as the plasmid is the most commonly used form of vector at present.

5 However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which are, or become, known in the art.

The "host cells" used in the present invention generally are procaryotic or eucaryotic hosts which preferably have been manipulated by the methods disclosed in EPO Publication No. 0130756 or 0251446 or U.S. Patent No. 5,371,008 to render them incapable of secreting enzymatically active endoprotease. A preferred host

10 cell for expressing subtilisin is the *Bacillus* strain BG2036 which is deficient in enzymatically active neutral protease and alkaline protease (subtilisin). The construction of strain BG2036 is described in detail in EPO Publication No. 0130756 and further described by Yang, M.Y., *et al.* (1984) *J. Bacteriol.* 160:15-21. Such host cells are distinguishable from those disclosed in PCT Publication No. 03949 wherein enzymatically inactive mutants of intracellular proteases in *E. coli* are disclosed. Other host cells for expressing subtilisin include

15 *Bacillus subtilis* var. I168 (EPO Publication No. 0130756).

Host cells are transformed or transfected with vectors constructed using recombinant DNA techniques. Such transformed host cells are capable of either replicating vectors encoding the subtilisin mutants or expressing the desired subtilisin mutant. In the case of vectors which encode the pre or prepro form of the subtilisin mutant, such mutants, when expressed, are typically secreted from the host cell into the host cell medium.

20 "Operably linked" when describing the relationship between two DNA regions simply means that they are functionally related to each other. For example, a presequence is operably linked to a peptide if it functions as a signal sequence, participating in the secretion of the mature form of the protein most probably involving cleavage of the signal sequence. A promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned

25 so as to permit translation.

The genes encoding the naturally-occurring precursor subtilisin may be obtained in accord with the general methods described in U.S. Patent No. 4,760,025 or EPO Publication No. 0130756. As can be seen from the examples disclosed therein, the methods generally comprise synthesizing labeled probes having putative sequences encoding regions of the hydrolase of interest, preparing genomic libraries from organisms expressing

30 the hydrolase, and screening the libraries for the gene of interest by hybridization to the probes. Positively hybridizing clones are then mapped and sequenced.

The cloned subtilisin is then used to transform a host cell in order to express the subtilisin. The subtilisin gene is then ligated into a high copy number plasmid. This plasmid replicates in hosts in the sense that it contains the well-known elements necessary for plasmid replication: a promoter operably linked to the gene in question

35 (which may be supplied as the gene's own homologous promotor if it is recognized, *i.e.*, transcribed, by the host), a transcription termination and polyadenylation region (necessary for stability of the mRNA transcribed by the host from the hydrolase gene in certain eucaryotic host cells) which is exogenous or is supplied by the endogenous terminator region of the subtilisin gene and, desirably, a selection gene such as an antibiotic resistance gene that enables continuous cultural maintenance of plasmid-infected host cells by growth in

antibiotic-containing media. High copy number plasmids also contain an origin of replication for the host, thereby enabling large numbers of plasmids to be generated in the cytoplasm without chromosomal limitations. However, it is within the scope herein to integrate multiple copies of the subtilisin gene into host genome. This is facilitated by procaryotic and eucaryotic organisms which are particularly susceptible to homologous recombination.

Once the subtilisin gene has been cloned, a number of modifications are undertaken to enhance the use of the gene beyond synthesis of the naturally-occurring precursor subtilisin. Such modifications include the production of recombinant subtilisin as disclosed in U.S. Patent No. 5,371,008 or EPO Publication No. 0130756 and the production of subtilisin mutants described herein.

10 *Mutant design and preparation.*

A. Subtilisin Variants Capable of Cleaving Substrates Having Dibasic Residues.

For the preparation of subtilisin variants capable of cleaving substrates containing dibasic residues, the following analysis was undertaken.

A number of structures have been solved of subtilisin with a variety of inhibitors and transition state analogs bound (Wright, C. S., Alden, R. A. and Kraut, J. (1969) *Nature*, 221:235-242; McPhalen, C.A. and James, N.G. (1988) *Biochemistry*, 27:6582-6598; Bode, W., Papamokos, E., Musil, D., Seemueller, U. and Fritz, M. (1986) *EMBO J.*, 5:813-818; and Bott, R., Utsch, M., Kossiakoff, A., Graycar, T., Katz, B. and Power, S. (1988) *J. Biol. Chem.*, 263:7895-7906). One of these structures, Figure 1, was used to locate residues that are in close proximity to side chains at the P1 and P2 positions from the substrate. Previous work had shown that replacement residues at positions 156 and 166 in the S1 binding site with various charged residues lead to improved specificity for complementary charged substrates (Wells, J. A., Powers, D. B., Bott, R. R., Graycar, T. P. and Estell, D. A. (1987) *Proc. Natl. Acad. Sci. USA*, 84:1219-1223). Although longer range electrostatic effects of substrate specificity have been noted (Russell, A. J. and Fersht, A. R. (1987) *Nature*, 328:496-500) these were generally much smaller than local ones. Therefore, it seemed reasonable that local differences in charge between subtilisin BPN' and the eukaryotic enzymes may account for the differences in specificity.

A detailed sequence alignment of 35 different subtilisin-like enzymes (Siezen, R. J., de Vos, W. M., Leunissen, A. M., and Dijkstra, B. W. (1991) *Prot. Eng.*, 4:719-737) allowed us to identify differences between subtilisin BPN' and the eukaryotic processing enzymes, KEX2, furin and PC2. Within the S1 binding pocket there are a number of charged residues that appear in the pro-hormone processing enzymes and not in subtilisin BPN' (Table 1A).

TABLE 1A

S1 subsite

	125-131 ^a	151-157	163-168
Subtilisin BPN'	SLGGPSG (SEQ ID NO: 3)	AAAGNEG (SEQ ID NO: 4)	ST-VGY P (SEQ ID NO: 5)
Kex2	SWGPA DD (SEQ ID NO: 6)	FASGN GG (SEQ ID NO: 7)	CNYDGY T (SEQ ID NO: 8)
Furin	SWGPE DD (SEQ ID NO: 9)	WASGN GG (SEQ ID NO: 10)	CNCDGY T (SEQ ID NO: 11)
PC2	SWGPA DD (SEQ ID NO: 6)	WASGD GG (SEQ ID NO: 12)	CNCDGY A (SEQ ID NO: 13)

^a numbering according to subtilisin BPN' sequence

For example, the eukaryotic enzymes have two conserved Asp residues at 130 and 131 as well as an Asp at 165 that is preceded by insertion of a Tyr or Cys. However, in the region from 151-157, subtilisin BPN' contains a Glu and the eukaryotes a conserved Gly.

In the S2 binding site there were two notable differences in sequence (Table 1B).

TABLE 1B

S2 subsite

	30-35	60-64
Subtilisin BPN'	V I D S G I (SEQ ID NO: 14)	D N N S H (SEQ ID NO: 15)
KEX2	I V D D G L (SEQ ID NO: 16)	S D D Y H (SEQ ID NO: 17)
Furin	I L D D G I (SEQ ID NO: 18)	N D N R H (SEQ ID NO: 19)
PC2	I M D D G I (SEQ ID NO: 20)	W F N S H (SEQ ID NO: 21)

Subtilisin BPN' contains a Ser at position 33 whereas the pro-hormone processing enzymes contain Asp. There is not as clear a consensus in the region of 60-64, but one notable difference is at position 62. This side chain which points directly at the P2 side chain (Figure 1) is Asn in subtilisin BPN', furin and PC2 but Asp in KEX2. Thus, not all substitutions were clearly predictive of the specificity differences.

A variety of mutants were produced to probe and engineer the specificity of subtilisin BPN' using oligonucleotides described in Table 2.

TABLE 2
Oligonucleotides used for site-directed
mutagenesis on subtilisin.

Mutant	Oligonucleotide	Specificity Pocket	Activity Expressed
5	S33D 5'- GCGGTTATCGACG*A*CGGTATCGATTCT -3' (SEQ ID NO: 22)	S2	+
	S33K 5'- GCGGTTATCGACAA*A*G*GTATCGATTCT -3' (SEQ ID NO: 23)	S2	+
	S33E 5'- GCGGTTATCGACG*A*A*GGTATCGATTCT -3' (SEQ ID NO: 24)	S2	+
	N62D 5'- CCAAGACAACG*ACTCTCACGGAA -3' (SEQ ID NO: 25)	S2	+
	N62S 5'- CCAAGACAACAG*CTCTCACGGAA -3' (SEQ ID NO: 26)	S2	+
10	N62K 5'- CCAAGACAACAAA*TCTCACGGAA -3' (SEQ ID NO: 27)	S2	+
	G166D 5'-CACTTCCGGCAGCTCG*T*C*G*ACAGTGA*C*T ACCCTGGC.AAATA-3' (SEQ ID NO: 28) (Inserts Sal I site)	S1	+
	G166E 5'-CACTTCCGGCAGCTCG*T*C*G*ACAGTGA*GT ACCCTGGCAAATA-3' (SEQ ID NO: 29) (Inserts Sal I site)	S1	+
	G128P/P129A 5'- TTAACATGAGCCTCGGCC*C*AG*CTA*G*C*GGT TCTGCTGCTTTA -3' (SEQ ID NO: 30) (Inserts Nhe I site)	S1	-
15	G128P/P129A/ S130D/G131D 5'- TTAACATGAGCCTCGGCC*C*C*G*CGG*A*TGA* TTCTGCTGCTTTAAA -3' (SEQ ID NO: 31) (Inserts Sac II site)	S1	-
	T164N/V165D 5'-CGGCAGCTCAAGCA*A*C*G*A*T*GGCTAT*CCT GGCAAATACCCTTCTGTCA -3' (SEQ ID NO: 32) (Inserts BsaBI site)	S1	-

T164Y/V165D	5'-CGGCAGCTCAAGCA*A*C*G*A*T*GGCTAT*CCT GGCAAATACCCTTCTGTCA -3' (SEQ ID NO: 33) (Inserts BsaBI site)	S1	-
T164N- Y(insert)- V165D	5'-ACTTCCGGCAGCTCT*T*C*G*AA*C*T*A*C*G*A* C*GGGTACCCTGGCAAATA-3' (SEQ ID NO: 34) (Inserts BstBI site)	S1	-
N62D/G166D	See individual mutations	S1/S2	+
N62D/G166E	See individual mutations	S1/S2	+

* Asterisks indicate base changes from the pSS5 (wild-type) template.

After producing the mutant plasmids they were transformed into a protease deficient strain of *B. subtilis* (BG2036) that lacks an endogenous gene for secretion of subtilisin. These were then tested for protease activity on skim milk plates.

The first set of mutants tested were ones where segments of the S1 binding site were replaced with sequences from KEX2. None of these segment replacements produced detectable activity on skim milk plates even though variants of subtilisin whose catalytic efficiencies are reduced by as much as 1000-fold do produce detectable halos (Wells, J.A., Cunningham, B.C., Graycar, T.P. and Estell, D.A. (1986) *Philos. Trans. R. Soc. Lond. A* . 317:415-423). We went on to produce single residue substitutions that should have less impact on the stability. These mutants at positions 166 in the S1 site, and 33 and 62 in the S2 site, were chosen based on the modeling and sequence considerations described above. Fortunately all single mutants as well as combination mutants produced activity on skim milk plates and could be purified to homogeneity.

Kinetic analysis of variant subtilisins.

To probe the effects of the G166E and G166D on specificity at the P1 position we used substrates having the form suc-AAPX-pna (SEQ ID NO: 69) where X was either Lys (SEQ ID NO. 58), Arg (SEQ ID NO. 59), Phe (SEQ ID NO. 56), Met (SEQ ID NO. 60) or Gln (SEQ ID NO. 61). The k_{cat}/K_m values were determined from initial rate measurements and results reported in Figure 2. Whereas the wild-type enzyme preferred Phe>Met>Lys>Arg>Gln, the G166E preferred Lys>Phe>Arg>Met>Gln, and G166D preferred Lys>Phe>Arg>Met>Gln. Thus, both the acidic substitutions at position 166 caused a shift in preference for basic residues at the P1 site, as previously reported (Wells, J. A., Powers, D. B., Bott, R. R., Graycar, T. P. and Estell, D. A. (1987a), *Proc. Natl. Acad. Sci. USA* 84:1219-1223).

The effects of single and double substitutions in the S2 binding site were analyzed with substrates having the form, suc-Ala-Ala-Xaa-Phe-pna (SEQ ID NO. 70) and are shown in Figure 3. At the P2 position the wild-type enzyme preferred Ala>Pro>Lys>Arg>Asp. In contrast, the S33D preferred Ala>Lys>Arg>Pro>Asp and the N62D preferred Lys>Ala>Arg>Pro>Asp. Although the effects were more dramatic for the N62D mutant, the S33D variant also showed significant improvement toward basic P2 residues and corresponding reduction

in hydrolysis of the Ala and Asp P2 substrates. We then analyzed the double mutant, but found it exhibited the catalytic efficiency of the worse of the two single mutants for each of the substrates tested.

Despite the less than additive effects seen for the two charged substitutions in the S2 site, we decided to combine the best S2 site variant (N62D) with either of the acidic substitutions in the S1 site. The two double mutants, N62D/G166E and N62D/G166D, were analyzed with substrates having the form, suc-AAXX-pna (SEQ ID NO. 71) where XX was either KK (SEQ ID NO. 66), KR (SEQ ID NO. 67), KF (SEQ ID NO. 62), PK (SEQ ID NO. 58), PF (SEQ ID NO. 56) or AF (SEQ ID NO. 63) (Figure 4). The wild-type preference was AF>PF>KF>KK>PK>KR, whereas the double mutants had the preference KK>KR>KF>PK>AF>PF. Thus for the double mutants there was a dramatic improvement toward cleavage of dibasic substrates and away from cleaving the hydrophobic substrates.

The greater than additive effect (or synergy) of these mutants can be seen from ratios of the catalytic efficiencies for the single and multiple mutants. For example, the G166E variant cannot distinguish Lys from Phe at the P1 position. Yet the N62D/G166E variant cleaves the Lys-Lys substrate about 8 times faster than the Lys-Phe substrate. Similarly the G166D cleaves the Lys P1 substrate about 3 times faster than the Phe P1 substrate, but the N62D/G166D double mutant cleaves a Lys-Lys substrate 18 times faster than a Lys-Phe substrate. Thus, as opposed to the reduction in specificity seen for the double mutant in the S2 site, the S1-S2 double mutants enhance specificity for basic residues. It is possible that these two sites bind the dibasic substrates in a cooperative manner analogous to a chelate effect.

Therefore, according to the present invention, subtilisin mutants having a preference for dibasic residues are preferred. According to this aspect of the present invention substitution of amino acids corresponding to amino acids N62 and G166 of subtilisin BPN' produced from *Bacillus amyloliquefaciens* are prepared. In particular, amino acids 62 and 166, or their equivalents, in the precursor subtilisin are substituted with amino acid residues Asp or Glu. Preferred subtilisin variants according to this aspect of the invention include N62D/G166D, N62E/G166E, N62E/G166D, and N62D/G166E variants of subtilisin BPN' and their equivalents.

25 B. Subtilisin Variants Capable of Cleaving Substrates Having Tribasic Residues

For the preparation of subtilisin variants specific for substrates containing a third basic residue at substrate position P4 we used the crystal structure of subtilisin BPN' complexed with Ala-Ala-Pro-Phe-Boronate (SEQ ID NO: 56) (Figure 7) in combination with sequence alignments of subtilisin BPN', KEX2, Furin, PC2, and P (Table 3) in designing basic specificity into the S1 and S2 and S4 subsites. The two subtilisin BPN' residues that most prominently display their side chains into the S4 pocket are Y104 and I107 (Figure 7).

Sequence alignments of subtilisin BPN' and the mammalian prohormone-processing proteases (Siezen, R. J., de Vos, W. M., Leunissen, A. M., and Dijkstra, B. W. (1991) *Prot. Eng.* 4:719-737) (Table 3) reveal that position 104 is conserved as Asp, and 107 as Glu in the prohormone converting (Arg-P4 specific) enzymes. Therefore these two mutations were introduced either individually or in combination into the dibasic-specific N62D/G166D subtilisin BPN' background (Table 4).

Table 3 Sequence alignments for the S4 site of subtilisins

S4 Site 100-110			
5	Subtilisin	GSGQYSWIING	(SEQ ID NO: 77)
	KEX2	GDITTEDEAAS	(SEQ ID NO: 78)
	Furin	GEVTDAREARS	(SEQ ID NO: 79)
	PC2	PFMTDIEEASS	(SEQ ID NO: 80)
	P	GIVTDAREASS	(SEQ ID NO: 81)

10 Table 4 describes oligonucleotides used for site-directed mutagenesis, protein regions affected by the mutations, and relative expression of protein for N62D/G166D subtilisin BPN' variants. Bold type indicates base changes from the pSS5 (N62D/G166D) template. For "Protein Expressed," "+" indicates a high level of expression of mature enzyme in crude culture medium, and "-" indicates no enzyme detectable.

TABLE 4

15	K it	<u>Oligonucleotide</u>	<u>Protein Region</u>	<u>Pro- tein Ex- pres- sed</u>
	Y104D	5'- GGTTCGGCCAA.GATAGCTGGATCATT -3' (SEQ ID NO: 82)	S4 pocket	-
	I107E	5'- CCAATACAGCTGGGAAATTAACGGAATCG -3' (SEQ ID NO: 83)	S4 pocket	-
	Y104D/I107E	5'- GGTTCGGCCAAAGATAGCTGGGAAATTAACG GAATCGA -3' (SEQ ID NO: 84)	S4 pocket	-
20	A(-4)R/ A(-2)K/ Y(-1)R	5'- AAGAAGATCACGTAAGACATAAGCGCGCGC AGTCCGTGC -3' (SEQ ID NO: 85)	Proces- sing site	-
25	Y104D/ A(-4)R/ A(-2)K/ Y(-1)R	See individual mutations	S4 pocket + Proces- sing site	+
	I107E/ A(-4)R/ A(-2)K/ Y(-1)R	See individual mutations	S4 pocket + Proces- sing site	-
30	Y104D/I107E/ A(-4)R/ A(-2)K/ Y(-1)R	See individual mutations	S4 pocket + Proces- sing site	-

Initial attempts to express the triple mutants in *Bacillus* were unsuccessful, as indicated by SDS-PAGE of crude supernatants. We reasoned that the source of the expression problem could lie in the fact that correct folding and maturation of subtilisin requires autolytic cleavage of its propeptide (Power, S.D., Adams, R. M., and Wells, J. A. (1986) *Proc. Natl. Acad. Sci. USA* 83, 3096-3100). The processing site in the wild-type enzyme has a sequence that is optimized for the natural substrate preference, AHAYIA (1 denotes the site of cleavage). Although the N62D/G166D subtilisin can still autolyze itself with the wild-type processing site, the additional S4 pocket mutations could reduce the cleavage to the point where expression was lowered to a minute level.

To test whether the mutants were expressed poorly due to an inability to autolytically process itself, mutations in the processing site were simultaneously incorporated to accommodate the changes in substrate specificity. Thus the sequence from positions -4 to -1 was changed from AHAY to RHKR in combination with the S4 site mutations. For N62D/Y104D/G166D, high levels of expression could then be achieved providing an indication that the additional Y104D mutation induced an especially strong preference for P4 Arg over Ala. Variants containing the I107E mutation, however, could not be expressed even with the change in the processing site.

Kinetic analysis of variant subtilisins

The mature N62D/Y104D/G166D variant was purified and analyzed for its ability to hydrolyze several tetrapeptide-pNA substrates. Table 5 displays the results along with data for the N62D/G166D mutant and wild-type subtilisin.

Table 5

Kinetic analysis of WT, N62D/G166D, and N62D/Y104D/G166D subtilisin BPN' mutants versus succinyl-tetrapeptide-pNA substrates. Kinetic constants were determined from plots of initial rates versus substrate concentration. Units are as follows: k_{cat} , s^{-1} ; K_m , μM ; and k_{cat}/K_m , $M^{-1}s^{-1}$. Standard errors were less than 15%.

Tetrapeptide Sequence, P4-P3-P2-P1												
	AAPF			AAKR			RAKR			RAKR		
	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m
Mutant												
WT	29	110	2.6×10^3	2.8	1700	1.7×10^{3a}	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
N62D/ G166D	3.4	180	1.9×10^3	15	41	3.7×10^3	N.D. ^b	N.D. ^b	N.D. ^b	N.D.	N.D.	N.D.
N62D/ Y104D/ G166D	-	-	8.0×10^{0c}	0.27	34	8.1×10^3	11	23	4.9×10^3	9.8	29	3.4×10^3

^a Artificially low k_{cat}/K_m presumably due to competing cleavage between Lys and Arg

^b Biphasic reaction progress curves presumably due to multiple cleavages in the substrate

^c Unable to saturate the enzyme, apparent k_{cat}/K_m calculated from rates at low substrate concentrations assuming

$$v = (k_{cat}/K_m) (E) (S)$$

The tribasic substrates succinyl-RAKR-pNA (SEQ ID NO: 86) and succinyl-KAKR-pNA (SEQ ID NO: 87) were hydrolyzed with high catalytic efficiency (k_{cat}/K_m) by the triple mutant, at a level similar to wild-type subtilisin versus one of its best substrates, succinyl-AAPF-pNA (SEQ ID NO: 56). In contrast, the dibasic substrate succinyl-AAKR-pNA (SEQ ID NO: 67) was hydrolyzed 60-fold less efficiently, mostly
5 due to diminution of k_{cat} . This indicates a dramatic specificity change from the wild-type preference at P4, at which hydrophobic residues are strongly favored over charged side chains (Grøn, H. and Breddam, K. (1992) *Biochemistry* 31, 8967-8971). In fact N62D/G166D subtilisin appears to cleave at an alternate site in the succinyl-RAKR-pNA (SEQ ID NO: 86) substrate, indicating that Arg was not accepted in its wild-type S4 site.

10 The large magnitude of the combined specificity changes in the N62D/Y104D/G166D variant is evidenced by its strong discrimination against substrates that are preferred by the wild-type enzyme. For example, succinyl-AAPF-pNA (SEQ ID NO: 56) is hydrolyzed 6×10^4 -fold less efficiently than succinyl-RAKR-pNA (SEQ ID NO: 86). Clearly, the S4 site mutation greatly improves upon the discriminatory power of the parent dibasic-specific N62D/G166D subtilisin, where the ratio of catalytic efficiency for
15 succinyl-AAKR-pNA versus succinyl-AAPF-pNA is 1.9×10^2 . The improvement in discrimination (310-fold) is also higher than would be predicted from the data for hydrolysis of succinyl-RAKR-pNA (SEQ ID NO: 86) versus succinyl-AAKR-pNA (SEQ ID NO: 67) by the triple mutant (a 60-fold effect).

Therefore in order to produce subtilisin variants capable of cleaving substrates containing basic residues at positions P4, P2, and P1, additional site specific substitutions are made in the dibasic specific
20 subtilisin variants. According to this aspect of the invention, substitution of the amino acid corresponding to Y104 of subtilisin BPN' produced by *Bacillus Amyloliquefaciens*, i.e., amino acid 104 of subtilisin BPN' or its equivalent, produces a variant having substantially altered substrate specificity. In a preferred embodiment of the present invention amino acids corresponding to N62, Y104, and G166 of subtilisin BPN' are substituted with acidic amino acids, preferably Asp and Glu and most preferably Asp. Subtilisin BPN'
25 variants N62D/Y104D/G166D, N62D/Y104E/G166D, N62E/Y104D/G166E, N62E/Y104E/G166E, N62E/Y104D/G166D, N62E/Y104E/G166D, N62D/Y104E/G166E, and N62D/Y104D/G166E, and there equivalents are preferred. Most preferred among this group of subtilisin variants are the N62D/Y104D/G166D subtilisin BPN' variants and their equivalents.

Mutagenesis and Synthetic Techniques

30 Various techniques are available which may be employed to produce mutant DNA, which can encode the subtilisin variants of the present invention. For instance, it is possible to derive mutant DNA based on naturally occurring DNA sequences that encode for changes in an amino acid sequence of the resultant protein relative to a precursor subtilisin. These mutant DNA can be used to obtain the variants of the present invention.

35 According to the invention, specific residues of *B. amyloliquefaciens* subtilisin are identified for substitution. These amino acid residue position numbers refer to those assigned to the *B. amyloliquefaciens* subtilisin sequence (see the mature sequence in Fig. 1. of U.S. Patent No. 4,760,025). The invention, however, is not limited to the mutation of this particular subtilisin but extends to precursor subtilisins

containing amino acid residues which are equivalent, as defined herein, to the particular identified residues in *B. amyloliquefaciens* subtilisin. Equivalent amino acids can be found in, for instance, subtilisin Carlsberg from *Bacillus licheniformis*, Bode et al., (1986) EMBO J., 5:813-818; thermolysin from *Thermolysin*, Gros et al., (1989) J. Mol. Biol. 210:347-367; and proteinase K from *Tritirachium album*, Betzel, et al., (1988) Acta Crystallogr., B, 44:163-172 as described by Siezen et al., (1991) Prof. Eng., 4: 719-737.

By way of illustration, with expression vectors encoding the precursor subtilisin in hand (see for example U.S. Patent No 4,760,025) site specific mutagenesis (Kunkel et al., (1991) Methods Enzymol. 204:125-139; Carter, P., et al., (1986) Nucl. Acids. Res. 13:4331; Zoller, M. J. et al., (1982) Nucl. Acids Res. 10:6487), cassette mutagenesis (Wells, J. A., et al., (1985) Gene 34:315), restriction selection
10 mutagenesis (Wells, J. A., et al., (1986) Philos. Trans. R. Soc. London Ser A 317, 415) or other known techniques may be performed on the DNA. The mutant DNA can then be used in place of the parent DNA by insertion into the appropriate expression vectors. Growth of host bacteria containing the expression vectors with the mutant DNA allows the production of variants which can be isolated as described herein.

Oligonucleotide-mediated mutagenesis is a preferred method for preparing the variants of the present
15 invention. This technique is well known in the art as described by Adelman et al., (1983) DNA, 2:183. Briefly, the native or unaltered DNA of a precursor subtilisin, for instance subtilisin BPN', is altered by hybridizing an oligonucleotide encoding the desired mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of the precursor.

20 After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the DNA.

Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the
25 nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques known in the art such as those described by Crea et al. (1987) Proc. Natl. Acad. Sci. USA, 75:5765. Exemplary oligonucleotide sequences for introducing amino acid changes into precursor subtilisin BPN' are provided in Tables 2 and 4.

30 Single-stranded DNA template may also be generated by denaturing double-stranded plasmid (or other) DNA using standard techniques.

For alteration of the native DNA sequence (to generate amino acid sequence variants, for example), the oligonucleotide is hybridized to the single-stranded template under suitable hybridization conditions. A DNA polymerizing enzyme, usually the Klenow fragment of DNA polymerase I, is then added to
35 synthesize the complementary strand of the template using the oligonucleotide as a primer for synthesis. A heteroduplex molecule is thus formed such that one strand of DNA encodes the variant form of the subtilisin, and the other strand (the original template) encodes the native, unaltered sequence of the precursor subtilisin. This heteroduplex molecule is then transformed into a suitable host cell. After the cells are grown, they are plated onto agarose plates and screened using the oligonucleotide primer radiolabeled with 32-phosphate to

identify the bacterial colonies that contain the mutated DNA. The mutated region is then removed and placed in an appropriate vector for protein production, generally an expression vector of the type typically employed for transformation of an appropriate host.

The method described immediately above may be modified such that a homoduplex molecule is created wherein both strands of the plasmid contain the mutation(s). The modifications are as follows: The single-stranded oligonucleotide is annealed to the single-stranded template as described above. A mixture of three deoxyribonucleotides, deoxyriboadenosine (dATP), deoxyriboguanosine (dGTP), and deoxyribothymidine (dTTP), is combined with a modified thio-deoxyribocytosine called dCTP-(α S) (which can be obtained from Amersham Corporation). This mixture is added to the template-oligonucleotide complex. Upon addition of DNA polymerase to this mixture, a strand of DNA identical to the template except for the mutated bases is generated. In addition, this new strand of DNA will contain dCTP-(α S) instead of dCTP, which serves to protect it from restriction endonuclease digestion.

After the template strand of the double-stranded heteroduplex is nicked with an appropriate restriction enzyme, the template strand can be digested with ExoIII nuclease or another appropriate nuclease past the region that contains the site(s) to be mutagenized. The reaction is then stopped to leave a molecule that is only partially single-stranded. A complete double-stranded DNA homoduplex is then formed using DNA polymerase in the presence of all four deoxyribonucleotide triphosphates, ATP, and DNA ligase. This homoduplex molecule can then be transformed into a suitable host cell as described above.

DNA encoding variants with more than one amino acid to be substituted may be generated in one of several ways. If the amino acids are located close together in the polypeptide chain, they may be mutated simultaneously using one oligonucleotide that codes for all of the desired amino acid substitutions. If, however, the amino acids are located some distance from each other (separated by more than about ten amino acids), it is more difficult to generate a single oligonucleotide that encodes all of the desired changes. Instead, one of two alternative methods may be employed.

In the first method, a separate oligonucleotide is generated for each amino acid to be substituted. The oligonucleotides are then annealed to the single-stranded template DNA simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired amino acid substitutions.

The alternative method involves two or more rounds of mutagenesis to produce the desired mutant. The first round is as described for the single mutants: wild-type DNA is used for the template, an oligonucleotide encoding the first desired amino acid substitution(s) is annealed to this template, and the heteroduplex DNA molecule is then generated. The second round of mutagenesis utilizes the mutated DNA produced in the first round of mutagenesis as the template. Thus, this template already contains one or more mutations. The oligonucleotide encoding the additional desired amino acid substitution(s) is then annealed to this template, and the resulting strand of DNA now encodes mutations from both the first and second rounds of mutagenesis. This resultant DNA can be used as a template in a third round of mutagenesis, and so on.

Cleavage of a Fusion Proteins With Subtilisin Variants

A fusion protein is any polypeptide that contains within it an affinity domain (AD) that usually aids in protein purification, a protease cleavage sequence or substrate linker (SL), which is cleaved by a protease and a protein product of interest (PP). Such fusion proteins are generally expressed by recombinant DNA technology. The genes for fusion proteins are designed so that the SL is between the AD and PP. These usually take the form AD-SL-PP such that the domain closest to the N-terminus is AD and PP is closest to the C-terminus.

Examples of AD would include, glutathione-S-transferase which binds to glutathione, protein A (or derivatives or fragments thereof) which binds IgG molecules, poly-histidine sequences, particularly (His)₆ (SEQ ID NO: 51) that bind metal affinity columns, maltose binding protein that binds maltose, human growth hormone that binds the human growth hormone receptor or any of a variety of other proteins or protein domains that can bind to an immobilized affinity support with an association constant (K_a) of $>10^5 M^{-1}$.

The SL can be any sequence which is cleaved by the subtilisin variants of the present invention. In preparations where the variant N62D/Y104D/G166D or its equivalent are used the SL can be any sequence, preferably at least 4 amino acids, in which the P4, P2, and P1 residues are basic residues. Therefore a SL linker is employed of the general formula P4-P3-P2-P1 wherein P4, P2, and P1 are basic amino acid residues. Preferred SLs according to this aspect of the invention include Lys-Ala-Lys-Arg (SEQ ID NO: 87) and Arg-Ala-Lys-Arg (SEQ ID NO: 86).

Likewise, where the N62D/G166D subtilisin variant is contemplated the SL preferably contains dibasic residues. For the variants capable of cleaving substrates containing dibasic residues the SL should be at least four residues and preferably contain a large hydrophobic residue at P4 (such as Leu or Met) and dibasic residues at P2 and P1 (such as Arg and Lys). A particularly good substrate is Leu-Met-Arg-Lys (SEQ ID NO: 52), but a variety of other sequences may work including Ala-Ser-Arg-Arg (SEQ ID NO: 50) and even Leu-Thr-Ala-Arg (SEQ ID NO 53).

It is often useful that the SL contain a flexible segment on its N-terminus to better separate it from the AD and PP. Such sequences include Gly-Pro-Gly-Gly (SEQ ID NO: 54) but can be as simple as Gly-Gly or Pro-Gly. Thus, an example of a particularly good SL would have the sequence Gly-Pro-Gly-Gly-Leu-Met-Arg-Lys (SEQ ID NO: 88) in the case of subtilisin variants capable of cleaving substrates containing dibasic amino acids, or Gly-Pro-Gly-Gly-Lys-Ala-Lys-Arg (SEQ ID NO: 89). This sequence would be inserted between the AD and PP domains.

The PP can be virtually any protein or peptide of interest but preferably should not have a Pro, Ile, Thr, Val, Asp or Glu as its first residue (P1'), or Pro or Gly at the second residue (P2') or Pro at the third residue (P3'). Such residues are poor substrates for the enzyme and may impair the ability of the subtilisins variant to cleave the SL sequence.

The conditions for cleaving the fusion protein are best done in aqueous solution, although it should be possible to immobilize the enzyme and cleave the soluble fusion protein. It may also be possible to cleave the fusion protein as it remains immobilized on a solid support (e.g. bound to the solid support through AD) with the soluble subtilisin variant. It is preferable to add the enzyme to the fusion protein so that the enzyme

is less than one part in 100 (1:100) by weight. A good buffer is 10-50mM Tris (pH 8.2) in 10mM NaCl. A preferable temperature is about 25°C although the enzyme is active up to 65°C. The extent of cleavage can be assayed by applying samples to SDS-PAGE. Generally suitable conditions for using the subtilisin variants of this invention do not depart substantially from those known in the art for the use of other subtilisins.

EXAMPLES

In the examples below and elsewhere, the following abbreviations are employed: subtilisin BPN', subtilisin from *Bacillus amyloliquefaciens*; Boc-RVRR-MCA (SEQ ID NO. 73), N-t-butoxy carbonyl-arginine-valine-arginine-arginine-7-amido-4-methyl coumarin; suc-Ala-Ala-Pro-Phe-pna (SEQ ID NO. 56), N-succinyl-alanine-alanine-proline-phenylalanyl-p-nitroanalide (SEQ ID NO. 56); hGH, human growth hormone; hGHbp, extracellular domain of the hGH receptor; PBS, phosphate buffered saline; AP, alkaline phosphatase;

Example 1

Construction and Purification of Subtilisin Mutants.

Site-directed mutations were introduced into the subtilisin BPN' gene cloned into the phagemid pSS5 (Wells, J. A., Ferrari, E., Henner, D. J., Estell, D.A. and Chen, E. Y. (1983) *Nucl. Acids Res.* 11:7911-7929). Single-stranded uracil-containing pSS5 template was prepared and mutagenesis performed using the method of Kunkel (Kunkel, T. A., Bebenek, K and McClary, J. (1991) *Methods Enzymol.* 204:125-139). For example, the synthetic oligonucleotide N62D,

(5'- CCAAGACAACG*ACTCTCACGGAA -3') (SEQ ID NO. 25)

in which the asterisk denotes a mismatch to the wild-type sequence, was used to construct the N62D mutant. The oligonucleotide was first phosphorylated at the 5' end using T4 polynucleotide kinase according to a described procedure (Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in "Molecular Cloning: A Laboratory Manual," Second Edition, Cold Spring Harbor, N.Y.). The phosphorylated oligonucleotide was annealed to single-stranded uracil-containing pSS5 template, the complementary DNA strand was filled in with deoxynucleotides using T7 polynucleotide kinase, and the resulting nicks ligated using T4 DNA ligase according to a previously described procedure (Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in "Molecular Cloning: A Laboratory Manual," Second Edition, Cold Spring Harbor, N.Y.). Heteroduplex DNA was transformed into the E. coli host JM101 (Yanish-Perron, C., Viera, J., and Messing, J. (1985) *Gene* 33: 103-199), and putative mutants were confirmed by preparation and dideoxy nucleotide sequencing of single stranded DNA (Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* 74:5463-5467) according to the SEQUENASE® protocol (USB Biochemicals). Mutant single-stranded DNA was then retransformed into JM101 cells and double stranded DNA prepared according to a previously

described procedure (Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in "Molecular Cloning: A Laboratory Manual," Second Edition, Cold Spring Harbor, N.Y.). For other mutations also requiring the use of one primer, the oligonucleotides used are listed in Table 2. For several of these oligonucleotides, additional silent mutations emplacing new restriction sites were simultaneously introduced to provide an
5 alternative verification of mutagenesis.

To construct the double mutants N62D/G166D, and N62D/G166E, pSS5 DNA containing the N62D mutation was produced in single-stranded uracil-containing form using the Kunkel procedure (Kunkel, T. A., Bebenek, K and McClary, J. (1991) *Methods Enzymol.* 204, 125-139). This mutant DNA was used as template for the further introduction of the G166D or G166E mutations, using the appropriate
10 oligonucleotide primers (see sequences in Table 2), following the procedures described above.

To construct the triple mutants, such as N62D/Y104D/G166D, pSS5 DNA containing the N62D/G166D mutation or other appropriate double mutation, was produced in single-stranded uracil-containing form using the Kunkel procedure (Kunkel, T. A., Bebenek, K and McClary, J. (1991) *Methods Enzymol.* 204, 125-139). This mutant DNA was used as template for the further introduction of the Y104D
15 mutations, using the appropriate oligonucleotide primers (see sequences in Table 4), following the procedures described above.

For expression of the subtilisin BPN' mutants, double stranded mutant DNA was transformed into a protease-deficient strain (BG2036) of *Bacillus Subtilis* (Yang, M. Y., Ferrari, E. and Henner, D. J. (1984) *Journal of Bacteriology* 160:15-21) according to a previous method (Anagnostopolous, C. and
20 Spizizen, J. (1961) *Journal of Bacteriology* 81:741-746) in which transformation mixtures were plated out on LB plus skim milk plates containing 12.5 µg/mL chloramphenicol. The clear halos indicative of skim milk digestion surrounding transformed colonies were noted to roughly estimate secreted protease activity.

The transformed BG2036 strains were cultured by inoculating 5 mL of 2xYT media (Miller, J. H., (1972) in "Experiments in Molecular Genetics," Cold Spring Harbor, N.Y.) containing 12.5 µg/mL
25 chloramphenicol and 2 mM CaCl₂ at 37 °C for 18-20 h, followed by 1:100 dilution in the same medium and growth in shake flasks at 37 °C for 18-22 h with vigorous aeration. The cells were harvested by centrifugation (6000g, 15 min, 4°C), and to the supernatant 20mM (final) CaCl₂ and one volume of ethanol (-20°C) were added. After 30 min at 4°C, the solution was centrifuged (12,000g, 15 min, 4°C), and one volume of ethanol (-20°C) added to the supernatant. After 2 h at -20°C, the solution was centrifuged
30 (12,000g, 15 min, 4°C) and the pellet resuspended in and dialyzed against MC (25 mM 2-(N-Morpholino)ethanesulfonic acid (MES), 5 mM CaCl₂ at pH 5.5) overnight at 4°C. The dialysate was passed through a 0.22 µm syringe filter and loaded onto a mono-S cation exchange column run by an FPLC system (Pharmacia Biotechnology). The column was washed with 20 volumes of MC and mutant subtilisin eluted over a linear gradient of zero to 0.15 M NaCl in MC, all at a flow rate of 1 mL/min. Peak fractions were
35 recovered and the subtilisin mutant quantitated by measuring the absorbance at 280 nm (E_{280} 0.1% = 1.17) (Matsubara, H.; Kasper, C B.; Brown, D. M.; and Smith, E. L. (1965) *J. Biol. Chem.*, 240:1125-1130.)

Example 2

Kinetic Characterizations

Subtilisins were assayed by measuring the initial rates of hydrolysis of *p*-nitroanilide tetrapeptide substrates in 0.4 mL 20 mM Tris-Cl pH 8.2, 4 % (v/v) dimethyl sulfoxide at $(25 \pm 0.2)^\circ\text{C}$ as described previously (Estell, D. A., Graycar, T. P., Miller, J. V., Powers, D. B., Burnier, J. P., Ng, P. G. and Wells, J. A. (1986)*Science* 233:659-663). Enzyme concentrations (E_0) were determined spectrophotometrically using $E_{280\text{ nm}} 0.1\% = 1.17$ (Matsubara, H.; Kasper, C B.; Brown, D. M.; and Smith, E. L. (1965)*J. Biol. Chem.* 240:1125-1130.), and were typically 5-50 nM in reactions. Initial rates were determined for nine to twelve different substrate concentrations over the range of 0.001-2.0 mM. Plots of initial rates (v) versus substrate concentration (S) were fitted to the Michaelis-Menton equation,

$$v = \frac{k_{cat}(E)_0((S))}{K_m + (S)}$$

to determine the kinetic constants k_{cat} and K_m (Fersht, A. in "Enzyme Structure and Mechanism", Second edition, Freeman and Co., N.Y.) using the program Kaleidagraph (Synergy Software, Reading, PA).

Example 3

Substrate Phage

Substrate phage selections were performed as described by Matthews and Wells (Matthews, D. J. and Wells, J. A. (1993)*Science* 260:1113-1117), with minor modifications. Phage sorting was carried out using a library in which the linker sequence between the gene III coat protein and a tight-binding variant of hGH was GPGGX₃GGPG (SEQ ID NO. 52). The library contained 2×10^6 independent transformants. Phage particles were prepared by infecting 1 mL of log phase 27C7 ($F'/\text{tet}^R/\text{OmpT}^+/\text{degP}^-$) *Escherichia coli* with approximately 10^8 library phage for 1 h at 37°C , followed by 18-24 h of growth in 25 mL 2YT medium containing 10^{10} M13K07 helper phage and 50 $\mu\text{g/mL}$ carbenicillin at 37°C . Wells of a 96-well Nunc Maxisorb microtiter plate were coated with 2 $\mu\text{g/mL}$ of hGHbp in 50 mM NaHCO_3 at pH 9.6 overnight at 4°C and blocked with PBS (10 mM sodium phosphate at pH 7.4 and 150 mM NaCl) containing 2.5% (w/v) skim milk for 1 h at room temperature. Between 10^{11} and 10^{12} phage in 0.1 mL 10 mM tris-Cl (pH 7.6), 1 mM EDTA, and 100 mM NaCl were incubated in the wells at room temperature for 2 h with gentle agitation. The plate was washed first with 20 rinses of PBS plus 0.05% Tween 20 and then twice with 20 mM tris-Cl at pH 8.2. The N62D/G166D subtilisin was added in 0.1 mL of 20 mM tris-Cl at pH 8.2 and protease sensitive phage were eluted after a variable reaction time. The concentration of protease and incubation times for elution of sensitive phage were decreased gradually over the course of sorting procedure to increase selectivity, with protease concentrations of 0.2 nM (rounds 1-3) and 0.1 nM (rounds 4-9), and reaction times of 5 min (rounds 1-6), 2.5 min (round 7), 40 s (round 8) and 20 s (round 9). Control wells in which no protease was added were also included in each round. For the resistant phage pool, the incubation time with protease remained constant at 5 min. The wells were then washed ten times with PBS plus 0.05% Tween 20 and resistant phage eluted by treatment with 0.1 mL of 0.2 M glycine at pH 2.0 in PBS plus 0.05% Tween

- 20 for 1 min at room temperature. Protease sensitive and resistant phage pools were titrated and used to infect log phase 27C7 cells for 1 h at 37°C, followed by centrifugation at 4000 rpm, removal of supernatant, and resuspension in 1 mL 2YT medium. The infected cells were then grown 18-24 h in the presence of helper phage as described above and the process repeated 9 times. Selected substrates were introduced into
- 5 AP fusion proteins and assayed for relative rates of cleavage as described by Matthews and Wells (Matthews, D. J., Goodman, L. J., Gorman, C. M., and Wells, J. A. (1994) *Protein Science* 3:1197-1205 and Matthews, D. J. and Wells, J. A. (1993) *Science* 260:1113-1117), except that the cleavage reactions were performed in 20 mM Tris-Cl at pH 8.2.

Example 4

10 Substrate phage selection and cleavage of a fusion protein

- Subtilisin has the capability to bind substrates from the P4 to P3' positions (McPhalen, C. A. and James, N. G. (1988) *Biochemistry* 27:6582-6598 and Bode, W., Papamokos, E., Musil, D., Seemueller, U. and Fritz, M. (1986) *EMBO J.* 5:813-818). Given this extensive binding site and the apparent cooperative nature in the way the substrate can bind the enzyme we wished to explore more broadly the substrate
- 15 preferences for the enzyme. To do this we utilized the substrate phage selection (Matthews, D. J., Goodman, L. J., Gorman, C. M., and Wells, J. A. (1994) *Protein Science* 3:1197-1205 and Matthews, D. J. and Wells, J. A. (1993) *Science* 260:1113-1117) described in Example 3. In this method a five-residue substrate linker that was flanked by di-glycine residues is inserted between an affinity domain (in this case a high affinity variant of hGH) and the carboxy-terminal domain of gene III, a minor coat protein displayed on the surface
- 20 of the filamentous phage, M13. The five residue substrate linker is fully randomized to generate a library of 20⁵ different protein sequence variants. These are displayed on the phage particles which are allowed to bind to the hGHbp. The protease of interest was added and if it cleaved the phage particle at the substrate linker it released that particle. The particles released by protease treatment can be propagated and subjected to another round of selection to further enrich for good protease substrates. Sequences that are retained can
- 25 also be propagated to enrich for poor protease substrates. By sequencing the isolated phage genes at the end of either selection one can identify good and poor substrates for further analysis.

- We chose to focus on the subtilisin BPN' variant N62D/G166D as it was slightly better at discriminating the synthetic dibasic substrates from the others. We subjected the substrate phage library to nine rounds of selection with the subtilisin variant and isolated clones that were either increasingly sensitive
- 30 or resistant to cleavage. Of twenty-one clones sequenced from the sensitive pool eighteen contained dibasic residues, eleven of which had the substrate linker sequence Asn-Leu-Met-Arg-Lys (SEQ ID NO: 35) (Table 6).

TABLE 6

Substrate phage sequences sensitive or resistant to N62D/G166D subtilisin from a GG-xxxxx-GG library after 9 rounds of selection^a.

Protease Sensitive Pool

5	<u>No Basic Sites (0)</u>	<u>Monobasic Sites (3)</u>	<u>Dibasic Sites (18)</u>
		N L T A R (3) (SEQ ID NO: 34)	N L M R K (11) (SEQ ID NO: 35) T A S R R (4) (SEQ ID NO: 36) L T R R S (SEQ ID NO: 37) A L S R K (SEQ ID NO: 38) L M L R K (SEQ ID NO: 39)

Protease Resistant Pool

	<u>No Basic Sites (7)</u>	<u>Monobasic Sites (2)</u>	<u>Dibasic Sites (1)</u>
	A S T H F (SEQ ID NO: 40)	Q K P N F (SEQ ID NO: 41)	R K P T H (SEQ ID NO: 42)
10	I Q Q Q Y (SEQ ID NO: 43)	R P G A M (SEQ ID NO: 44)	
	Q G E L P (SEQ ID NO: 47)		
15	A P D P T (SEQ ID NO: 46)		
	Q L L E H (SEQ ID NO: 47)		
	V N N N H (SEQ ID NO: 48)		
20	A Q S N L (SEQ ID NO: 49)		

^a Numbers in parentheses indicate the number of times a particular DNA sequence was isolated.

Three (3) of the sensitive sequences were monobasic, Asn-Leu-Thr-Ala-Arg (SEQ ID NO: 34). It is known that subtilisin has a preference for hydrophobic residues at the P4 position. If these and the other selected substrates were indeed cleaved after the last basic residue they all would have a Leu, Met or Ala at the P4 position. Almost no basic residues were isolated in the protease resistant pool and those that were had a Pro following the mono- or dibasic residue. It is known that subtilisin does not cleave substrates containing

Pro at the P1' position (Carter, P., Nilsson, B., Burnier, J., Burdick, D. and Wells, J. A. (1989) *Proteins: Struct., Funct., Genet.* 6:240-248). Thus, di-basic substrates were highly selected and these had the additional feature of Leu, Met or Ala at the P4 position.

Example 5

Cleavage of Substrate Linkers

5

We wished to analyze how efficiently the most frequently selected sequences were cleaved in the context of a fusion protein. For this we applied an alkaline phosphatase-fusion protein assay (Matthews, D. J., Goodman, L. J., Gorman, C. M., and Wells, J. A. (1994) *Protein Science* 3:1197-1205 and Matthews, D. J. and Wells, J. A. (1993) *Science* 260:1113-1117). The hGH substrate linker domains were excised from the
10 phage vector by PCR and fused in front of the gene for *E. coli* AP. The fusion protein was expressed and purified on an hGH receptor affinity column. The fusion protein was bound to the hGH receptor on a plate and treated with the subtilisin variant. The rate of cleavage of the fusion protein from the plate was monitored by collecting soluble fractions as a function of time and assaying for AP activity (Figure 5). The most frequently isolated substrate sequence, Asn-Leu-Met-Arg-Lys (SEQ ID NO: 35) was cleaved about ten times
15 faster than the next most frequently isolated clones (Thr-Ala-Ser-Arg-Arg (SEQ ID NO: 36) and Asn-Leu-Thr-Ala-Arg (SEQ ID NO: 34). The cleaved AP products were also recovered and subjected to N-terminal sequencing to determine the sites of cleavage (Figure 5), cleavage site denoted by I). In all three fusion proteins, this site was immediately following the dibasic or monobasic site according to the mutant subtilisin design. We also tested the dibasic sequence isolated from the resistant pool, namely Arg-Lys-Pro-Thr-His
20 (SEQ ID NO: 42). We observed no detectable cleavage above background for this substrate during the assay.

The present invention has of necessity been discussed herein by reference to certain specific methods and materials. It is to be understood that the discussion of these specific methods and materials in no way
25 constitutes any limitation on the scope of the present invention, which extends to any and all alternative materials and methods suitable for accomplishing the ends of the present invention.

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All references cited herein are expressly incorporated by reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Genentech, Inc.
- (ii) TITLE OF INVENTION: SUBTILISIN VARIANTS CAPABLE OF CLEAVING
SUBSTRATES CONTAINING BASIC RESIDUES
- (iii) NUMBER OF SEQUENCES: 89
- (iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Genentech, Inc.
(B) STREET: 460 Point San Bruno Blvd
(C) CITY: South San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080
- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WinPatin (Genentech)
- (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/398028
(B) FILING DATE: 03-MAR-1995
- (viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kubinec, Jeffrey S.
(B) REGISTRATION NUMBER: 36,575
(C) REFERENCE/DOCKET NUMBER: P0936P1PCT
- (ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 415/225-8228
(B) TELEFAX: 415/952-9881
(C) TELEX: 910/371-7168

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8119 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAATTCNGGT CTACTAAAAT ATTATTCCAT ACTATACAAT TAATACACAG 50

AATAATCTGT CTATTGGTTA TTCTGCAAAT GAAAAAAGG AGAGGATAAA 100

GA GTG AGA GGC AAA AAA GTA TGG ATC AGT TTG CTG TTT 138
Val Arg Gly Lys Lys Val Trp Ile Ser Leu Leu Phe
-107 -105 -100

GCT TTA GCG TTA ATC TTT ACG ATG GCG TTC GGC AGC ACA 177
Ala Leu Ala Leu Ile Phe Thr Met Ala Phe Gly Ser Thr

-95 -90 -85
 TCC TCT GCC CAG GCG GCA GGG AAA TCA AAC GGG GAA AAG 216
 Ser Ser Ala Gln Ala Ala Gly Lys Ser Asn Gly Glu Lys
 -80 -75 -70
 5 AAA TAT ATT GTC GGG TTT AAA CAG ACA ATG AGC ACG ATG 255
 Lys Tyr Ile Val Gly Phe Lys Gln Thr Met Ser Thr Met
 -65 -60
 10 AGC GCC GCT AAG AAG AAA GAT GTC ATT TCT GAA AAA GGC 294
 Ser Ala Ala Lys Lys Lys Asp Val Ile Ser Glu Lys Gly
 -55 -50 -45
 GGG AAA GTG CAA AAG CAA TTC AAA TAT GTA GAC GCA GCT 333
 Gly Lys Val Gln Lys Gln Phe Lys Tyr Val Asp Ala Ala
 -40 -35
 15 TCA GCT ACA TTA AAC GAA AAA GCT GTA AAA GAA TTG AAA 372
 Ser Ala Thr Leu Asn Glu Lys Ala Val Lys Glu Leu Lys
 -30 -25 -20
 AAA GAC CCG AGC GTC GCT TAC GTT GAA GAA GAT CAC GTA 411
 Lys Asp Pro Ser Val Ala Tyr Val Glu Glu Asp His Val
 -15 -10 -5
 20 GCA CAT GCG TAC GCG CAG TCC GTG CCT TAC GGC GTA TCA 450
 Ala His Ala Tyr Ala Gln Ser Val Pro Tyr Gly Val Ser
 1 5
 25 CAA ATT AAA GCC CCT GCT CTG CAC TCT CAA GGC TAC ACT 489
 Gln Ile Lys Ala Pro Ala Leu His Ser Gln Gly Tyr Thr
 10 15 20
 GGA TCA AAT GTT AAA GTA GCG GTT ATC GAC AGC GGT ATC 528
 Gly Ser Asn Val Lys Val Ala Val Ile Asp Ser Gly Ile
 25 30 35
 30 GAT TCT TCT CAT CCT GAT TTA AAG GTA GCA GGC GGA GCC 567
 Asp Ser Ser His Pro Asp Leu Lys Val Ala Gly Gly Ala
 40 45
 AGC ATG GTT CCT TCT GAA ACA AAT CCT TTC CAA GAC AAC 606
 Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Asn
 50 55 60
 35 GAC TCT CAC GGA ACT CAC GTT GCC GGC ACA GTT GCG GCT 645
 Asp Ser His Gly Thr His Val Ala Gly Thr Val Ala Ala
 65 70
 40 CTT AAT AAC TCA ATC GGT GTA TTA GGC GTT GCG CCA AGC 684
 Leu Asn Asn Ser Ile Gly Val Leu Gly Val Ala Pro Ser
 75 80 85
 GCA TCA CTT TAC GCT GTA AAA GTT CTC GGT GCT GAC GGT 723
 Ala Ser Leu Tyr Ala Val Lys Val Leu Gly Ala Asp Gly
 90 95 100
 45 TCC GGC CAA TAC AGC TGG ATC ATT AAC GGA ATC GAG TGG 762
 Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu Trp
 105 110
 GCG ATC GCA AAC AAT ATG GAC GTT ATT AAC ATG AGC CTC 801
 Ala Ile Ala Asn Asn Met Asp Val Ile Asn Met Ser Leu
 115 120 125

GGC GGA CCT TCT GGT TCT GCT GCT TTA AAA GCG GCA GTT 840
 Gly Gly Pro Ser Gly Ser Ala Ala Leu Lys Ala Ala Val
 130 135

5 GAT AAA GCC GTT GCA TCC GGC GTC GTA GTC GTT GCG GCA 879
 Asp Lys Ala Val Ala Ser Gly Val Val Val Val Ala Ala
 140 145 150

GCC GGT AAC GAA GGC ACT TCC GGC AGC TCG TCG ACA GTG 918
 Ala Gly Asn Glu Gly Thr Ser Gly Ser Ser Ser Thr Val
 155 160 165

10 GAC TAC CCT GGC AAA TAC CCT TCT GTC ATT GCA GTA GGC 957
 Asp Tyr Pro Gly Tyr Pro Ser Val Ile Ala Val Gly
 170 175

GCT GTT GAC AGC AGC AAC CAA AGA GCA TCT TTC TCA AGC 996
 Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser
 180 185 190

15 GTA GGA CCT GAG CTT GAT GTC ATG GCA CCT GGC GTA TCT 1035
 Val Gly Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser
 195 200

20 ATC CAA AGC ACG CTT CCT GGA AAC AAA TAC GGG GCG TAC 1074
 Ile Gln Ser Thr Leu Pro Gly Asn Lys Tyr Gly Ala Tyr
 205 210 215

AAC GGT ACC TCA ATG GCA TCT CCG CAC GTT GCC GGA GCG 1113
 Asn Gly Thr Ser Met Ala Ser Pro His Val Ala Gly Ala
 220 225 230

25 GCT GCT TTG ATT CTT TCT AAG CAC CCG AAC TGG ACA AAC 1152
 Ala Ala Leu Ile Leu Ser Lys His Pro Asn Trp Thr Asn
 235 240

ACT CAA GTC CGC AGC AGT TTA GAA AAC ACC ACT ACA AAA 1191
 Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Thr Lys
 245 250 255

30 CTT GGT GAT TCT TTC TAC TAT GGA AAA GGG CTG ATC AAC 1230
 Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn
 260 265

GTA CAG GCG GCA GCT CAG TA AAACATAAAA AACCGGCCTT 1270
 Val Gln Ala Ala Ala Gln
 270 275

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 CCATAATCGA CGGATGGCTC CCTCTGAAAA TTTTAACGAG AAACGGCGGG 1370
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 CCTGATACCG GGAGACGGCA TTCGTAATCG GATCCGGAAT TTGTAACGT 1520
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 TTAACCAATA GGCCGAAATC GGCAAAATCC CTTATAAATC AAAAGAATAG 1620
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45 AAAGAACGTG GACTCCAACG TCAAAGGGCG AAAAACCCTC TATCAGGGCT 1720

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5 ACCACCACAC CCGCCGCGCT TAATGCGCCG CTACAGGGCG CGTCCGGATC 1970
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10 CGGCGAGCAC ATGGAACGGG TTGGCATGGA TTGTAGGCGC CGCCCTATAC 2220
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15 CGCATCTCGG GCCGCGTTC TGGCGTTTTT CCATAGGCTC CGCCCCCTG 2470
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5 AAGATAATTC TTATAATCAG CACATGCATG TATTGGTATG TGTGGAACCA 5270
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GACAACCGGT GAGTGGAAGG TGGAAGTGAA TTTTGATAGC GAAGAGATTC 6120
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25 AGTGTATCAA ACTGCTAAAT CGGTAGAAGC CCAAACGTT CACGATGCGA 6270
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30 CCTTCAGGTT ATGACCATCT GTGCCAGTTC GTAATGTCTG GTCACCTTC 6520
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CCATTTTGAA CGATGACCTC TAATAATTGT TAATCATGTT GGTACGTAT 6670

TTATTAAC TT CTCCTAGTAT TAGTAATTAT CATGGCTGTC ATGGCGCATT 6720
AACGGAATAA AGGGTGTGCT TAAATCGGGC CATTITGCGT AATAAGAAAA 6770
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GCTCAAATCC TTTAAAAAAA CACAAAAGAC CACATTTTTT AATGTGGTCT 6970
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AGTGGGATAT TTTTAAATA TATATTTATG TTACAGTAAT ATTGACTTTT 7070
AAAAAGGAT TGATTCTAAT GAAGAAAGCA GACAAGTAAG CCTCCTAAAT 7120
10 TCACCTTAGA TAAAAATTTA GGAGGCATAT CAAATGAACT TTAATAAAAT 7170
TGATTTAGAC AATTGGAAGA GAAAAGAGAT ATTTAATCAT TATTTGAACC 7220
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CGAAACATAA AACAGAAGG ATATAAATTT TACCCTGCAT TTATTTTCTT 7320
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15 GCGACGGAGA GTTAGGTTAT TGGGATAAGT TAGAGCCACT TTATACAATT 7420
TTTGATGGTG TATCTAAAAC ATTCTCTGGT ATTTGGACTC CTGTAAAGAA 7470
TGACTTCAA GAGTTTTATG ATTTATACCT TTCTGATGTA GAGAAATATA 7520
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20 CAATAATAAT AGTAATTACC TTCTACCCAT TATTACAGCA GGAAAATTCA 7670
TTAATAAAGG TAATTCAATA TATTTACCGC TATCTTTACA GGTACATCAT 7720
TCTGTTTGTG ATGGTTATCA TGCAGGATG TTTATGAACT CTATTCAGGA 7770
ATTGTCAGAT AGGCCTAATG ACTGGCTTTT ATAATATGAG ATAATGCCGA 7820
CTGTACTTTT TACAGTCGGT TTTCTAATGT CACTAACCTG CCCCCTTAGT 7870
25 TGAAGAAGGT TTTTATATTA CAGCTCCAGA TCCATATCCT TCTTTTTCTG 7920
AACCGACTTC TCCTTTTTTCG CTTCTTTATT CCAATTGCTT TATTGACGTT 7970
GAGCCTCGGA ACCCNTATAG TGTGTTATAC TTTACTTGGA AGTGGTTGCC 8020
GGAAAGAGCG AAAATGCCTC ACATTTGTGC CACCTAAAAA GGAGCGATTT 8070
ACATATGAGT TATGCAGTTT GTAGAATGCA AAAAGTGAAA TCAGGATCN 8119

30 (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 382 amino acids
 - (B) TYPE: Amino Acid
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

	Val Arg Gly Lys Lys Val Trp Ile Ser Leu Leu Phe Ala Leu Ala	
	-107 -105 -100 -95	
5	Leu Ile Phe Thr Met Ala Phe Gly Ser Thr Ser Ser Ala Gln Ala	
	-90 -85 -80	
	Ala Gly Lys Ser Asn Gly Glu Lys Lys Tyr Ile Val Gly Phe Lys	
	-75 -70 -65	
	Gln Thr Met Ser Thr Met Ser Ala Ala Lys Lys Lys Asp Val Ile	
	-60 -55 -50	
10	Ser Glu Lys Gly Gly Lys Val Gln Lys Gln Phe Lys Tyr Val Asp	
	-45 -40 -35	
	Ala Ala Ser Ala Thr Leu Asn Glu Lys Ala Val Lys Glu Leu Lys	
	-30 -25 -20	
15	Lys Asp Pro Ser Val Ala Tyr Val Glu Glu Asp His Val Ala His	
	-15 -10 -5	
	Ala Tyr Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala	
	1 5 10	
	Pro Ala Leu His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val	
	15 20 25	
20	Ala Val Ile Asp Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys	
	30 35 40	
	Val Ala Gly Gly Ala Ser Met Val Pro Ser Glu Thr Asn Pro Phe	
	45 50 55	
25	Gln Asp Asn Asp Ser His Gly Thr His Val Ala Gly Thr Val Ala	
	60 65 70	
	Ala Leu Asn Asn Ser Ile Gly Val Leu Gly Val Ala Pro Ser Ala	
	75 80 85	
	Ser Leu Tyr Ala Val Lys Val Leu Gly Ala Asp Gly Ser Gly Gln	
	90 95 100	
30	Tyr Ser Trp Ile Ile Asn Gly Ile Glu Trp Ala Ile Ala Asn Asn	
	105 110 115	
	Met Asp Val Ile Asn Met Ser Leu Gly Gly Pro Ser Gly Ser Ala	
	120 125 130	
35	Ala Leu Lys Ala Ala Val Asp Lys Ala Val Ala Ser Gly Val Val	
	135 140 145	
	Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly Ser Ser Ser	
	150 155 160	
	Thr Val Asp Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala Val Gly	
	165 170 175	
40	Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val Gly	
	180 185 190	
	Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr	
	195 200 205	

Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala
 210 215 220
 Ser Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His
 225 230 235
 5 Pro Asn Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr
 240 245 250
 Thr Thr Lys Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile
 255 260 265
 Asn Val Gln Ala Ala Ala Gln
 10 270 275

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 7 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ser Leu Gly Gly Pro Ser Gly
1 5 7

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 7 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ala Ala Ala Gly Asn Glu Gly
1 5 7

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 6 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ser Thr Val Gly Tyr Pro
1 5 6

35 (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 7 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ser Trp Gly Pro Ala Asp Asp
1 5 7

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear
- 5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
Phe Ala Ser Gly Asn Gly Gly
1 5 7
- (2) INFORMATION FOR SEQ ID NO:8:
- 10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
15 Cys Asn Tyr Asp Gly Tyr Thr
1 5 7
- (2) INFORMATION FOR SEQ ID NO:9:
- 20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:
Ser Trp Gly Pro Glu Asp Asp
1 5 7
- (2) INFORMATION FOR SEQ ID NO:10:
- 25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:
30 Trp Ala Ser Gly Asn Gly Gly
1 5 7
- (2) INFORMATION FOR SEQ ID NO:11:
- 35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:
Cys Asn Cys Asp Gly Tyr Thr
1 5 7
- 40 (2) INFORMATION FOR SEQ ID NO:12:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Trp Ala Ser Gly Asp Gly Gly
1 5 7

(2) INFORMATION FOR SEQ ID NO:13:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

10 Cys Asn Cys Asp Gly Tyr Ala
1 5 7

(2) INFORMATION FOR SEQ ID NO:14:

- 15 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Val Ile Asp Ser Gly Ile
1 5 6

20 (2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Asp Asn Asn Ser His
1 5

(2) INFORMATION FOR SEQ ID NO:16:

- 30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

35 Ile Val Asp Asp Gly Leu
1 5 6

(2) INFORMATION FOR SEQ ID NO:17:

- 40 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Ser Asp Asp Tyr His
1 5

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Ile Leu Asp Asp Gly Ile
1 5 6

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Asn Asp Asn Arg His
1 5

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Ile Met Asp Asp Gly Ile
1 5 6

25 (2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Trp Phe Asn Ser His
1 5

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

40 GCGGTTATCG ACGACGGTAT CGATTCT 27

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs

(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

5 GCGGTTATCG ACAAAGGTAT CGATTCT 27

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: Nucleic Acid
10 (C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GCGGTTATCG ACGAAGGTAT CGATTCT 27

(2) INFORMATION FOR SEQ ID NO:25:

15 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CCAAGACAAC GACTCTCAG GAA 23

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
25 (B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CCAAGACAAC AGCTCTCAG GAA 23

30 (2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
35 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CCAAGACAAC AAATCTCAG GAA 23

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
40 (A) LENGTH: 42 base pairs

(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

5 CACTTCCGGC AGCTCGTCGA CAGTGGACTA CCCTGGCAAA TA 42

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: Nucleic Acid
10 (C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CACTTCCGGC AGCTCGTCGA CAGTGGAGTA CCCTGGCAAA TA 42

(2) INFORMATION FOR SEQ ID NO:30:

15 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TTAACATGAG CCTCGGCCCA GCTAGCGGTT CTGCTGCTTT A 41

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
25 (B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

TTAACATGAG CCTCGGCCCC GCGGATGATT CTGCTGCTTT AAA 43

30 (2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
35 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CGGCAGCTCA AGCAACGATG GCTATCCTGG CAAATACCCT TCTGTCA 47

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
40 (A) LENGTH: 44 base pairs

(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

5 ACTTCCGGCA GCTCTTCGAA CTACGACGGG TACCCTGGCA AATA 44

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
10 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Asn Leu Thr Ala Arg
1 5

(2) INFORMATION FOR SEQ ID NO:35:

15 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

20 Asn Leu Met Arg Lys
1 5

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
25 (B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Thr Ala Ser Arg Arg
1 5

30 (2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Leu Thr Arg Arg Ser
1 5

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
40 (A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Ala Leu Ser Arg Lys
1 5

(2) INFORMATION FOR SEQ ID NO:39:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

10 Leu Met Leu Arg Lys
1 5

(2) INFORMATION FOR SEQ ID NO:40:

- 15 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

Ala Ser Thr His Phe
1 5

(2) INFORMATION FOR SEQ ID NO:41:

- 20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

25 Gln Lys Pro Asn Phe
1 5

(2) INFORMATION FOR SEQ ID NO:42:

- 30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

Arg Lys Pro Thr His
1 5

35 (2) INFORMATION FOR SEQ ID NO:43:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

Ile Gln Gln Gln Tyr
1 5

(2) INFORMATION FOR SEQ ID NO:44:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear
- 5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:
Arg Pro Gly Ala Met
1 5
- (2) INFORMATION FOR SEQ ID NO:45:
- 10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:
Gln Gly Glu Leu Pro
15 1 5
- (2) INFORMATION FOR SEQ ID NO:46:
- 20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:
Ala Pro Asp Pro Thr
1 5
- (2) INFORMATION FOR SEQ ID NO:47:
- 25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:
Gln Leu Leu Glu His
30 1 5
- (2) INFORMATION FOR SEQ ID NO:48:
- 35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:
Val Asn Asn Asn His
1 5
- 40 (2) INFORMATION FOR SEQ ID NO:49:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

Ala Gln Ser Asn Leu
1 5

(2) INFORMATION FOR SEQ ID NO:50:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

10 Thr Ala Ser Arg Arg
1 5

(2) INFORMATION FOR SEQ ID NO:51:

- 15 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

His His His His His His
1 5 6

20 (2) INFORMATION FOR SEQ ID NO:52:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

Leu Met Arg Lys
1 4

(2) INFORMATION FOR SEQ ID NO:53:

- 30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

35 Leu Thr Ala Arg
1 4

(2) INFORMATION FOR SEQ ID NO:54:

- 40 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

Gly Pro Gly Gly
1 4

(2) INFORMATION FOR SEQ ID NO:55:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

Gly Leu Met Arg Lys
1 5

(2) INFORMATION FOR SEQ ID NO:56:

- 10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

15 Ala Ala Pro Phe
1 4

(2) INFORMATION FOR SEQ ID NO:57:

- 20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

Gly Pro Gly Gly Xaa Xaa Xaa Xaa Gly Gly Pro Gly
1 5 10 13

25 (2) INFORMATION FOR SEQ ID NO:58:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

Ala Ala Pro Lys
1 4

(2) INFORMATION FOR SEQ ID NO:59:

- 35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

40 Ala Ala Pro Arg
1 4

(2) INFORMATION FOR SEQ ID NO:60:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

Ala Ala Pro Met
1 4

5 (2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

Ala Ala Pro Gln
1 4

(2) INFORMATION FOR SEQ ID NO:62:

15 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

20 Ala Ala Lys Phe
1 4

(2) INFORMATION FOR SEQ ID NO:63:

25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

Ala Ala Ala Phe
1 4

(2) INFORMATION FOR SEQ ID NO:64:

30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

35 Ala Ala Arg Phe
1 4

(2) INFORMATION FOR SEQ ID NO:65:

40 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

Ala Ala Asp Phe

1 4

(2) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

Ala Ala Lys Lys
1 4

10 (2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

Ala Ala Lys Arg
1 4

(2) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

25 Ala Ala Lys Phe
1 4

(2) INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

Ala Ala Pro Xaa
1 4

(2) INFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

40 Ala Ala Xaa Phe
1 4

(2) INFORMATION FOR SEQ ID NO:71:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

5 Ala Ala Xaa Xaa Xaa
 1 5

(2) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 275 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala Pro Ala
 1 5 10 15

15 Leu His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val
 20 25 30

Ile Asp Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys Val Ala
 35 40 45

20 Gly Gly Ala Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp
 50 55 60

Asn Asp Ser His Gly Thr His Val Ala Gly Thr Val Ala Ala Leu
 65 70 75

Asn Asn Ser Ile Gly Val Leu Gly Val Ala Pro Ser Ala Ser Leu
 80 85 90

25 Tyr Ala Val Lys Val Leu Gly Ala Asp Gly Ser Gly Gln Tyr Ser
 95 100 105

Trp Ile Ile Asn Gly Ile Glu Trp Ala Ile Ala Asn Asn Met Asp
 110 115 120

30 Val Ile Asn Met Ser Leu Gly Gly Pro Ser Gly Ser Ala Ala Leu
 125 130 135

Lys Ala Ala Val Asp Lys Ala Val Ala Ser Gly Val Val Val Val
 140 145 150

Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly Ser Ser Ser Thr Val
 155 160 165

35 Asp Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala Val Gly Ala Val
 170 175 180

Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val Gly Pro Glu
 185 190 195

40 Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr Leu Pro
 200 205 210

Gly Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Ser Pro
 215 220 225

His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn
 230 235 240

Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Thr
245 250 255

Lys Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val
260 265 270

5 Gln Ala Ala Ala Gln
275

(2) INFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

Arg Val Arg Arg
1 4

15 (2) INFORMATION FOR SEQ ID NO:74:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 1146 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

GTG AGA GGC AAA AAA GTA TGG ATC AGT TTG CTG TTT 36
Val Arg Gly Lys Lys Val Trp Ile Ser Leu Leu Phe
-107 -105 -100

25 GCT TTA GCG TTA ATC TTT ACG ATG GCG TTC GGC AGC ACA 75
Ala Leu Ala Leu Ile Phe Thr Met Ala Phe Gly Ser Thr
-95 -90 -85

30 TCC TCT GCC CAG GCG GCA GGG AAA TCA AAC GGG GAA AAG 114
Ser Ser Ala Gln Ala Ala Gly Lys Ser Asn Gly Glu Lys
-80 -75 -70

AAA TAT ATT GTC GGG TTT AAA CAG ACA ATG AGC ACG ATG 153
Lys Tyr Ile Val Gly Phe Lys Gln Thr Met Ser Thr Met
-65 -60

35 AGC GCC GCT AAG AAG AAA GAT GTC ATT TCT GAA AAA GGC 192
Ser Ala Ala Lys Lys Lys Asp Val Ile Ser Glu Lys Gly
-55 -50 -45

GGG AAA GTG CAA AAG CAA TTC AAA TAT GTA GAC GCA GCT 231
Gly Lys Val Gln Lys Gln Phe Lys Tyr Val Asp Ala Ala
-40 -35

40 TCA GCT ACA TTA AAC GAA AAA GCT GTA AAA GAA TTG AAA 270
Ser Ala Thr Leu Asn Glu Lys Ala Val Lys Glu Leu Lys
-30 -25 -20

45 AAA GAC CCG AGC GTC GCT TAC GTT GAA GAA GAT CAC GTA 309
Lys Asp Pro Ser Val Ala Tyr Val Glu Glu Asp His Val
-15 -10 -5

AGA CAT AAG CGC GCG CAG TCC GTG CCT TAC GGC GTA TCA 348

Arg His Lys Arg Ala Gln Ser Val Pro Tyr Gly Val Ser
1 5

CAA ATT AAA GCC CCT GCT CTG CAC TCT CAA GGC TAC ACT 387
Gln Ile Lys Ala Pro Ala Leu His Ser Gln Gly Tyr Thr
5 10 15 20

GGA TCA AAT GTT AAA GTA GCG GTT ATC GAC AGC GGT ATC 426
Gly Ser Asn Val Lys Val Ala Val Ile Asp Ser Gly Ile
25 30 35

GAT TCT TCT CAT CCT GAT TTA AAG GTA GCA GGC GGA GCC 465
10 Asp Ser Ser His Pro Asp Leu Lys Val Ala Gly Gly Ala
40 45

AGC ATG GTT CCT TCT GAA ACA AAT CCT TTC CAA GAC AAC 504
Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Asn
50 55 60

GAC TCT CAC GGA ACT CAC GTT GCC GGC ACA GTT GCG GCT 543
15 Asp Ser His Gly Thr His Val Ala Gly Thr Val Ala Ala
65 70

CTT AAT AAC TCA ATC GGT GTA TTA GGC GTT GCG CCA AGC 582
20 Leu Asn Asn Ser Ile Gly Val Leu Gly Val Ala Pro Ser
75 80 85

GCA TCA CTT TAC GCT GTA AAA GTT CTC GGT GCT GAC GGT 621
Ala Ser Leu Tyr Ala Val Lys Val Leu Gly Ala Asp Gly
90 95 100

TCC GGC CAA GAT AGC TGG ATC ATT AAC GGA ATC GAG TGG 660
25 Ser Gly Gln Asp Ser Trp Ile Ile Asn Gly Ile Glu Trp
105 110

GCG ATC GCA AAC AAT ATG GAC GTT ATT AAC ATG AGC CTC 699
Ala Ile Ala Asn Asn Met Asp Val Ile Asn Met Ser Leu
115 120 125

GGC GGA CCT TCT GGT TCT GCT GCT TTA AAA GCG GCA GTT 738
30 Gly Gly Pro Ser Gly Ser Ala Ala Leu Lys Ala Ala Val
130 135

GAT AAA GCC GTT GCA TCC GGC GTC GTA GTC GTT GCG GCA 777
35 Asp Lys Ala Val Ala Ser Gly Val Val Val Val Ala Ala
140 145 150

GCC GGT AAC GAA GGC ACT TCC GGC AGC TCG TCG ACA GTG 816
Ala Gly Asn Glu Gly Thr Ser Gly Ser Ser Ser Thr Val
155 160 165

GAC TAC CCT GGC AAA TAC CCT TCT GTC ATT GCA GTA GGC 855
40 Asp Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala Val Gly
170 175

GCT GTT GAC AGC AGC AAC CAA AGA GCA TCT TTC TCA AGC 894
Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser
180 185 190

GTA GGA CCT GAG CTT GAT GTC ATG GCA CCT GGC GTA TCT 933
45 Val Gly Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser
195 200

ATC CAA AGC ACG CTT CCT GGA AAC AAA TAC GGG GCG TAC 972
Ile Gln Ser Thr Leu Pro Gly Asn Lys Tyr Gly Ala Tyr

(2) INFORMATION FOR SEQ ID NO:75:

20

(A) LENGTH: 382 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

25

Val Arg Gly Lys Lys Val Trp Ile Ser Leu Leu Phe Ala Leu Ala
-107 -105 -100 -95

[illegible]

Ala Gly Lys Ser Asn Gly Glu Lys Lys Tyr Ile Val Gly Phe Lys
-75 -70 -65

Gln Thr Met Ser Thr Met Ser Ala Ala Lys Lys Lys Asp Val Ile
-60 -55 -50

Ser Glu Lys Gly Gly Lys Val Gln Lys Gln Phe Lys Tyr Val Asp
-45 -40 -35

Ala Ala Ser Ala Thr Leu Asn Glu Lys Ala Val Lys Glu Leu Lys
-30 -25 -20

35 Lys Asp Pro Ser Val Ala Tyr Val Glu Glu Asp His Val Arg His
 -15 -10 -5

Lys Arg Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala
1 5 10

Pro Ala Leu His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val
40 15 20 25

Ala Val Ile Asp Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys
30 35 40

Val Ala Gly Gly Ala Ser Met Val Pro Ser Glu Thr Asn Pro Phe
45 50 55

45 Gln Asp Asn Asp Ser His Gly Thr His Val Ala Gly Thr Val Ala

	60		65		70
	Ala Leu Asn Asn Ser Ile Gly Val Leu Gly Val Ala Pro Ser Ala				
	75		80		85
5	Ser Leu Tyr Ala Val Lys Val Leu Gly Ala Asp Gly Ser Gly Gln				
	90		95		100
	Asp Ser Trp Ile Ile Asn Gly Ile Glu Trp Ala Ile Ala Asn Asn				
	105		110		115
	Met Asp Val Ile Asn Met Ser Leu Gly Gly Pro Ser Gly Ser Ala				
	120		125		130
10	Ala Leu Lys Ala Ala Val Asp Lys Ala Val Ala Ser Gly Val Val				
	135		140		145
	Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly Ser Ser Ser				
	150		155		160
15	Thr Val Asp Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala Val Gly				
	165		170		175
	Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val Gly				
	180		185		190
	Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr				
	195		200		205
20	Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala				
	210		215		220
	Ser Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His				
	225		230		235
25	Pro Asn Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr				
	240		245		250
	Thr Thr Lys Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile				
	255		260		265
	Asn Val Gln Ala Ala Ala Gln				
	270		275		

30 (2) INFORMATION FOR SEQ ID NO:76:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 5 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

Asn Arg Met Arg Lys
 1 5

(2) INFORMATION FOR SEQ ID NO:77:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 11 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly
 1 5 10 11

(2) INFORMATION FOR SEQ ID NO:78:

- 5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 11 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

10 Gly Asp Ile Thr Thr Glu Asp Glu Ala Ala Ser
 1 5 10 11

(2) INFORMATION FOR SEQ ID NO:79:

- 15 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 11 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

Gly Glu Val Thr Asp Ala Val Glu Ala Arg Ser
 1 5 10 11

(2) INFORMATION FOR SEQ ID NO:80:

- 20 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 11 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

25 Pro Phe Met Thr Asp Ile Ile Glu Ala Ser Ser
 1 5 10 11

(2) INFORMATION FOR SEQ ID NO:81:

- 30 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 11 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

Gly Ile Val Thr Asp Ala Ile Glu Ala Ser Ser
 1 5 10 11

35 (2) INFORMATION FOR SEQ ID NO:82:

- 40 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 base pairs
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

GGTTCGGCC AAGATAGCTG GATCATT 27

(2) INFORMATION FOR SEQ ID NO:83:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
5 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

CCAATACAGC TGGGAAATTA ACGGAATCG 29

- (2) INFORMATION FOR SEQ ID NO:84:
- (i) SEQUENCE CHARACTERISTICS:
10 (A) LENGTH: 31 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

15 GGTTCGGGCC AAGATAGCTG GGAAATTAAC G 31

- (2) INFORMATION FOR SEQ ID NO:85:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: Nucleic Acid
20 (C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

AAGAAGATCA CGTAAGACAT AAGCGCGCGC 30

- (2) INFORMATION FOR SEQ ID NO:86:
- (i) SEQUENCE CHARACTERISTICS:
25 (A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

30 Arg Ala Lys Arg
1 4

- (2) INFORMATION FOR SEQ ID NO:87:
- (i) SEQUENCE CHARACTERISTICS:
35 (A) LENGTH: 4 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

Lys Ala Lys Arg
1 4

- (2) INFORMATION FOR SEQ ID NO:88:
- (i) SEQUENCE CHARACTERISTICS:
40 (A) LENGTH: 8 amino acids

(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

5 Gly Pro Gly Gly Leu Met Arg Lys
1 5 8

(2) INFORMATION FOR SEQ ID NO:89:

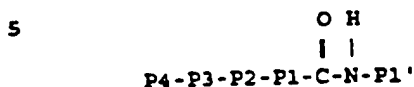
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
10 (B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

Gly Pro Gly Gly Lys Ala Lys Arg
1 5 8

What is claimed is:

1. A subtilisin variant derived from a precursor subtilisin-type serine protease said variant capable of cleaving a polypeptide substrate comprising the sequence:



wherein:

- P4 is a basic amino acid;
- 10 P3 is any amino acid selected from the naturally occurring amino acids;
- P2 is a basic amino acid;
- P1 is a basic amino acid; and
- P1' is not Pro.
2. The subtilisin variant of claim 1 containing an acidic amino acid at a residue equivalent to
- 15 Asn 62, Tyr 104 and Gly 166 of the subtilisin naturally produced by *Bacillus amyloliquefaciens*.
3. The subtilisin-type serine protease variant of claim 2 wherein the acidic amino acid is Asp or Glu.
4. The subtilisin-type serine protease variant of claim 3 wherein the acidic amino acid is Asp.
5. The subtilisin-type serine protease variant of claim 2 wherein the precursor subtilisin-type
- 20 serine protease in the subtilisin naturally produced by *Bacillus amyloliquefaciens*.
6. The subtilisin variant of claim 5 having the amino acid sequence of the mature polypeptide of Figure 8 (SEQ ID NO: 75).
7. A subtilisin variant having substrate specificity for peptide substrates containing dibasic amino acid sequences.
- 25 8. The subtilisin variant of claim 7 having a different amino acid residue at residue position +62 than subtilisin naturally produced by *Bacillus amyloliquefaciens*.
9. The subtilisin variant of Claim 8 having an Asp or Glu at residue position +62.
10. The subtilisin variant of Claim 9 having an Asp at residue position +62.
11. The subtilisin variant of Claim 10 further having an Asp or Glu at residue position +166.
- 30 12. The subtilisin variant of Claim 11 having an Asp at residue position +166.
13. The subtilisin variant of Claim 12 having the amino acid sequence of the mature polypeptide provided in Fig. 6.
14. An isolated nucleic acid molecule encoding the subtilisin variant of Claim 1.
15. The nucleic acid molecule of Claim 14 further comprising a promoter operably linked to the
- 35 nucleic acid molecule.
16. An expression vector comprising the nucleic acid molecule of Claim 15 operably linked to control sequences recognized by a host cell transformed with the vector.
17. A host cell transformed with the vector of Claim 16.
18. An isolated nucleic acid molecule encoding the subtilisin variant of Claim 7.

19. The nucleic acid molecule of Claim 18 further comprising a promoter operably linked to the nucleic acid molecule.
20. An expression vector comprising the nucleic acid molecule of Claim 19 operably linked to control sequences recognized by a host cell transformed with the vector.
- 5 21. A host cell transformed with the vector of Claim 20.
22. A process of using the nucleic acid molecule encoding the subtilisin variant to effect production of the subtilisin variant comprising culturing the host cell of Claim 21 under conditions suitable for expression of the subtilisin variant.
23. The process of Claim 22 further comprising recovering the subtilisin variant from the host
10 cell culture medium.
24. A method of using the subtilisin variant of Claim 1 comprising contacting a fusion protein containing a dibasic sequence with the subtilisin variant.
25. A process for cleaving a polypeptide, said polypeptide comprising an amino acid sequence represented by the formula:
- 15 P4-P3-P2-P1-P1'
- wherein,
- P4 is a basic amino acid;
- P3 is an amino acid selected from the naturally occurring amino acids;
- P2 is a basic amino acid;
- 20 P1 is a basic amino acid; and
- P1' is not Pro;
- comprising the step of:
- subjecting said polypeptide to the subtilisin variant of claim 1 in a reaction mixture under conditions such that the subtilisin variant cleaves the polypeptide.
- 25 26. A process of using the nucleic acid molecule encoding the subtilisin variant to effect production of the subtilisin variant comprising culturing the host cell of Claim 17 under conditions suitable for expression of the subtilisin variant.
27. The process of Claim 26 further comprising recovering the subtilisin variant from the host cell culture medium.
- 30 28. A method of using the subtilisin variant of Claim 7 comprising contacting a fusion protein containing a dibasic sequence with the subtilisin variant.
29. A process for cleaving a polypeptide, said polypeptide comprising an amino acid sequence represented by the formula:
- P4-P3-P2-P1-P1'
- 35 wherein,
- P4 is a large hydrophobic amino acid;
- P3 is an amino acid selected from the naturally occurring amino acids;
- P2 is a basic amino acid;
- P1 is a basic amino acid; and

PI' is not Pro;
comprising the step of:
subjecting said polypeptide to the subtilisin variant of claim 7 in a reaction mixture under conditions
such that the subtilisin variant cleaves the polypeptide.

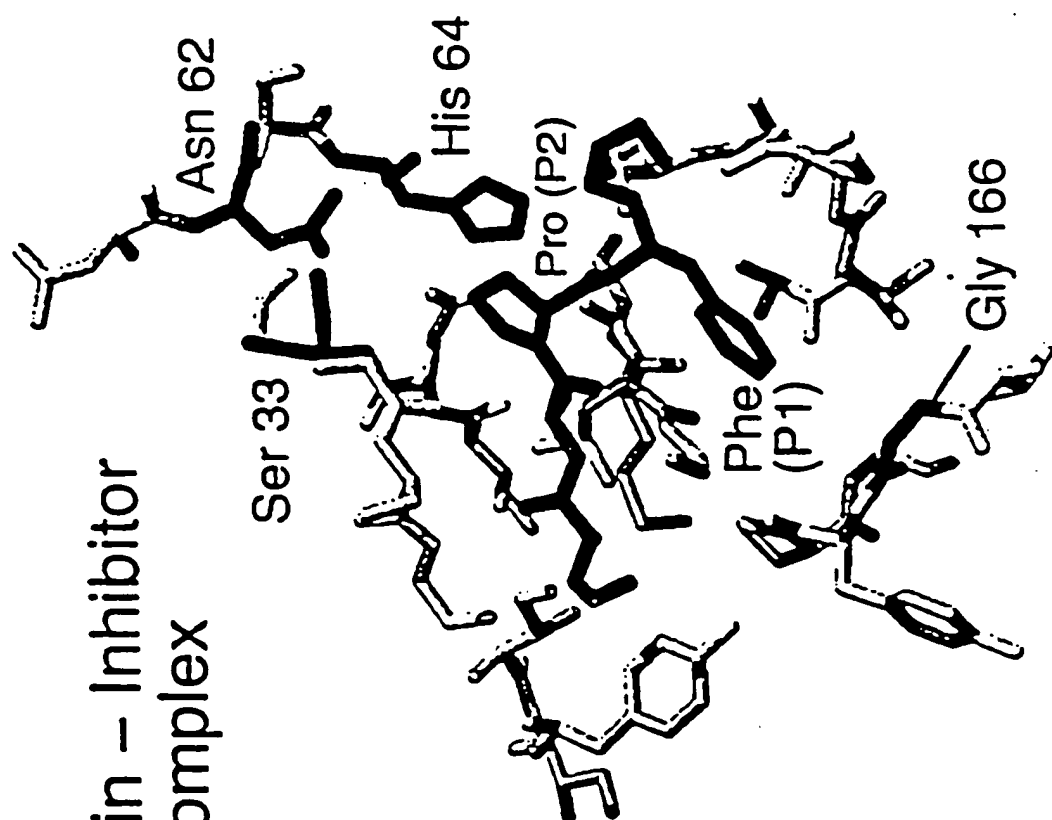


Figure 1

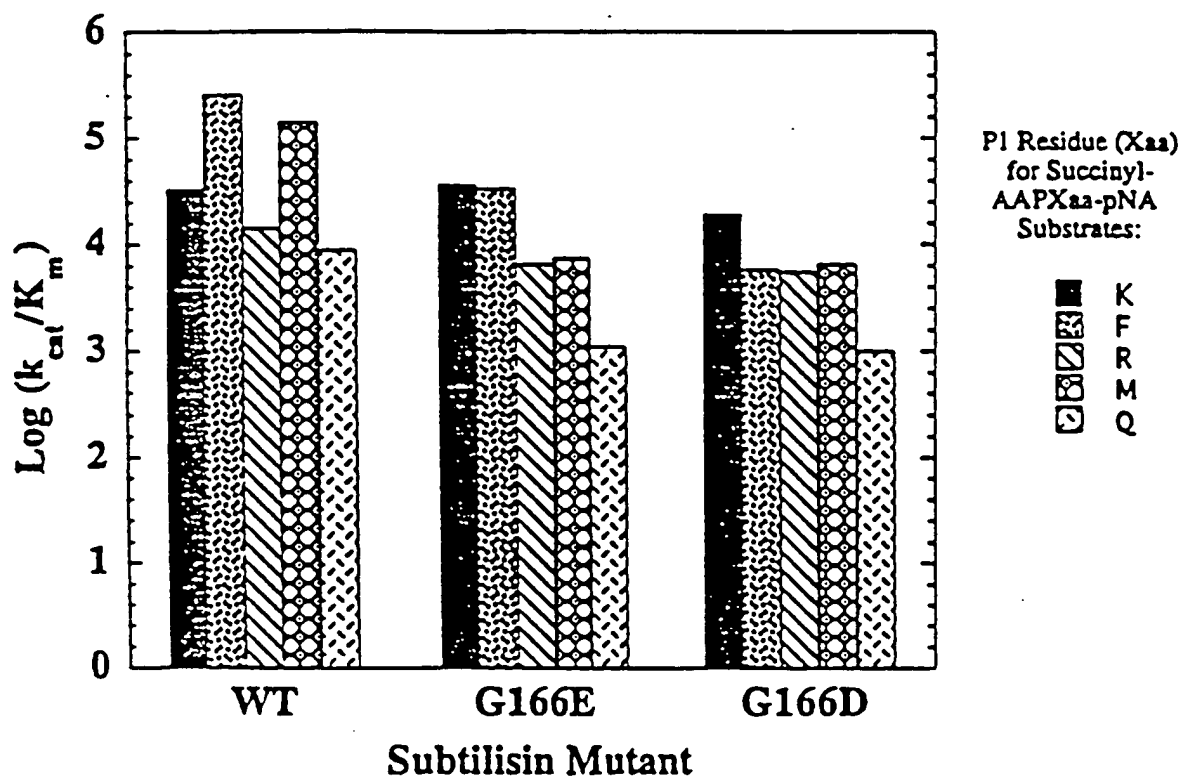


Figure 2

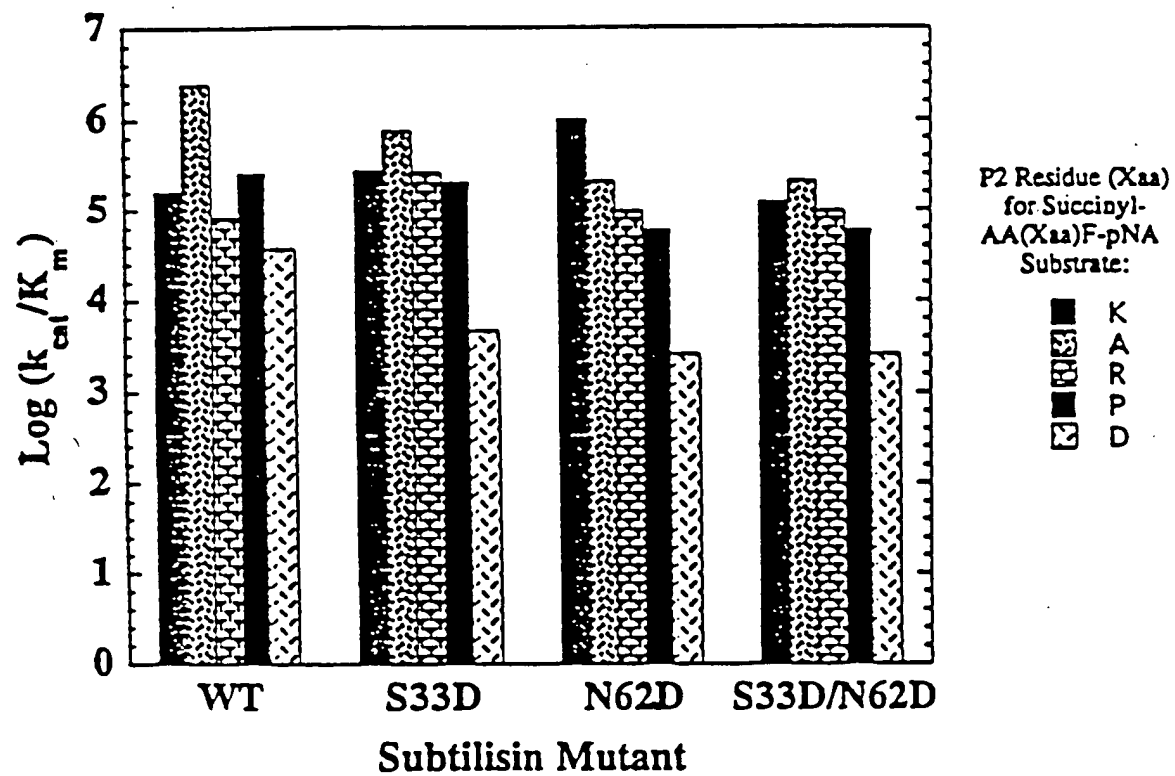


Figure 3

4/17

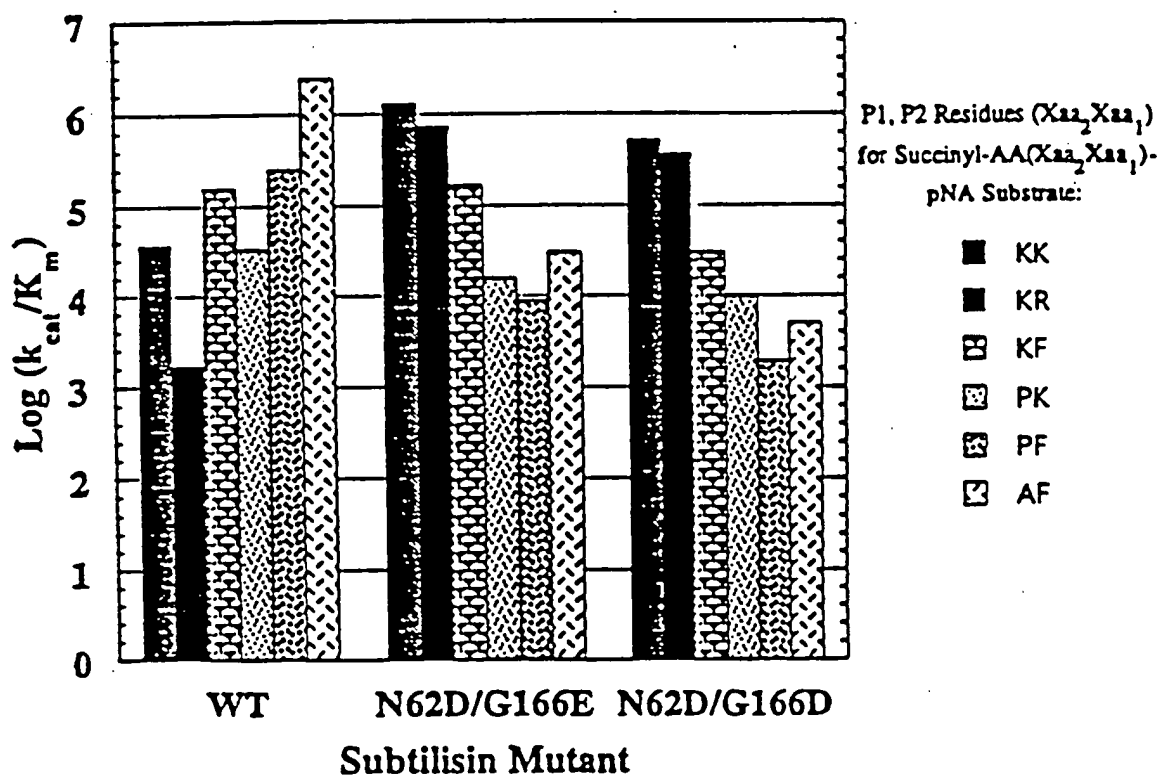


Figure 4

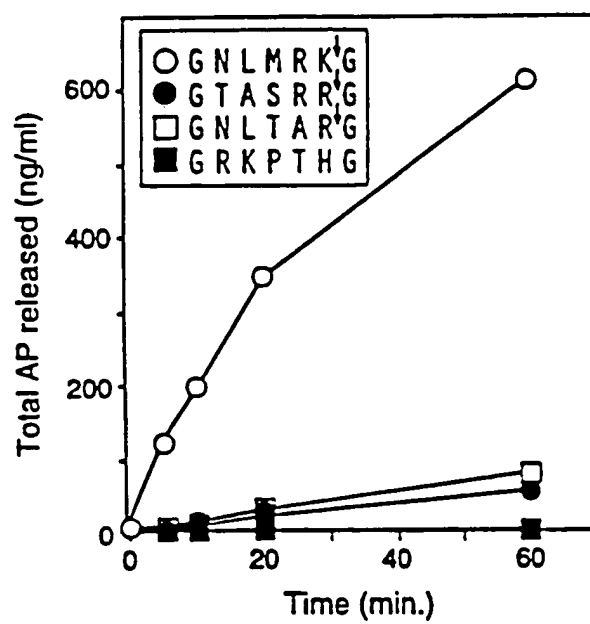


FIGURE 5

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ecorI      accI      sspI      aacI/aacII/vspI
1  GAATTCAGCT CTACCTAAAT ATTATTCAT ACTATCAAT TAATACACAG AATATCTCT CTATTCCTTA TTCTCCAAAT GAAATACAG AACATATA
CTTAAGCCCA GATCATTTTA TAATACGTA TCAATACCTA ATTAATCTCT TTATTAACA CATACCAAT AACAGCTTA CTTTTCCTC TCTCTAATT

101 GA  GTG AGA GCG AAA AAA GTA TCG ATC AGT TTG CTG TTT GCT TTA GCG TTA ATC TTT AGC ATG GCG TTC GCG AGC ACA TCC
CT  CAC TCT CCG TTT TTT CAT ACC TAC TCA AAC GAC AAA GCA AAT CCG AAT TAG AAA TGC TAC GCG AAG CCG TCG TGT AGC
Val Arg Gly Lys Lys Val Trp Ile Ser Leu Leu Phe Ala Leu Ala Leu Ile Phe Thr Met Ala Phe Gly Ser Thr Ser
-100

181 TCT GCG CAG GCG GCA GCG AAA TCA AAC GCG GAA AAG AAA TAT ATT CTC GCG TTT AAA CAG ACA ATG AGC AGC ATG AGC GCG
AGA CCG CTC CCG CCG TTT AGT TTG CCG CTT TTC TTT ATA TAA CAG CCG AAA TTT CTC TGT TAC TCG TCG TAC TCG CCG
Ser Ala Gln Ala Ala Gly Lys Ser Asn Gly Glu Lys Tyr Ile Val Gly Phe Lys Gln Thr Met Ser Thr Met Ser Ala
-80

bgII      paeI      ahoIII/dreI      bglAI/eepII      bspI286      bolIIAI      bayI      haeII
363 GCT AAG AAG AAA GAT GTC ATT TCT GAA AAA GCG GCG AAA GTG CAA AAG CAA TTC AAA TAT GTA GAC GCA GCT TCA GCT ACA
CGA TTC TTC TTT GTA CAG TAA ACA CTT TTT CCG CCG TTT CAC GCT TTC GGT AAG TTT ATA CAT CTG CGT GCA AAT GCA TGT
Ala Lys Lys Lys Asp Val Ile Ser Glu Lys Gly Gly Lys Val Gln Lys Gln Phe Lys Tyr Val Asp Ala Ala Ser Ala Thr
-50

363 GCT AAG AAG AAA GAT GTC ATT TCT GAA AAA GCG GCG AAA GTG CAA AAG CAA TTC AAA TAT GTA GAC GCA GCT TCA GCT ACA
CGA TTC TTC TTT GTA CAG TAA ACA CTT TTT CCG CCG TTT CAC GCT TTC GGT AAG TTT ATA CAT CTG CGT GCA AAT GCA TGT
Ala Lys Lys Lys Asp Val Ile Ser Glu Lys Gly Gly Lys Val Gln Lys Gln Phe Lys Tyr Val Asp Ala Ala Ser Ala Thr
-50

363 TTA AAC GAA AAA GCT GTA AAA GAA TTG AAA AAA GAC CCG ACC GTC GCT TAC GGT GAA GAA CAT GAC GCA GCA CAT GCG TAG
AAT TGC CTT TTT CGA CAT TTT CTT AAC TTT CTC GCG TCG CAG GCA ATG CAA CTT CTT GTA GTG CAT CCG GTA CCG ATG
Leu Asn Glu Lys Ala Val Lys Glu Leu Lys Lys Asp Pro Ser Val Ala Tyr Val Glu Glu Asp His Val Ala His Ala Tyr
-30

424 GCG CAG TCC CTG CCT TAC GCG GTA TCA CAA ATT AAA GCG CCG CCG GCT CTG CAC TCT CAA GCG TAC ACT GCA TCA AAT GTT AAA
CGC CTC AGC CAC AAG CCA ATG CCG CAT AGT GTT TAA TTT CCG GCA GCA GAC GTG ACA GGT CCG ATG TCA CCG AGT TTA CAA TTT
Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala Pro Ala Leu His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys
10

503 GTA GCG GTT ATC GAC AGC GGT ATC GAT TCT TCT GAT GCT GAT TTA AAG GTA GCA GCG GCA GCG ACC AGC ATG GGT CCT TCT GAA
CAT CCG CAA TAG CTG TCG CCA TAG CTA ACA ACA GCA GCA CTA AAT TTC CAT CGT CCG CCG TCG TAC CAA GCA ACA CAT
Val Ala Val Ile Asp Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys Val Ala Gly Gly Ala Ser Met Val Pro Ser Glu
30

366 ACA AAT CCT TTC CAA GAC AAG GAC TCT CAC GCA ACT CAC GGT CCG GCG ACA GGT CCG GCG CTT AAT AAG TCA ATG GAT GTA
TGT TTA GCA AAG GGT TGT CTA GCA GCG CCG CCG TCA GCA GCG CCG TGT CAA CCG GCA GAA TTA TGT AGT TAG CCA CAT
Thr Asn Pro Phe Gln Asp Asn Asp Ser His Gly Thr His Val Ala Gly Thr Val Ala Ala Leu Asn Asn Ser Ile Gly Val
60

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Figure 6-1

7/17

WO 96/27671

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667 TTA GGC GTT GCG CCA AGC GCA TCA CTT TAC GCT GTA AAA GTT CTC GGT GCT GAC GGT TCG GCG CAA TAC AGC TCG ATC ATT
    AAT CCG CAA GCG GGT TCG TCG CGT AGT GAA ATG GCA CAT TTT CAA GAG CCA GCA CTG CCA AGC GCG GTT ATG TCG ACC TAG TAA
    Leu Gly Val Ala Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu Gly Ala Asp Gly Ser Gly Gln Tyr Ser Trp Ile Ile
    90
    eaeI          pvuII
    offI          napBI
748 AAC GCA ATC GAG TCG GCG ATC GCA AAC AAT ATG GAC GTT ATT AAC ATG AGC CTC GCG GGA CCT TCT GGT TCT GCT GCT TTA
    TTG CCT TAG CTC ACC GCG TAG CCG TTT TAC TTA TAC CAG CAA TAA TTG TAC TCG GAG CCG CCG CCG CCA ACA CCA CCA AAT
    Asn Gly Ile Glu Trp Ala Ile Ala Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly Pro Ser Gly Ser Ala Ala Leu
    110
    pvuI/bspCI
    accI          shalI/draI
839 AAA GCG GCA GTT CAT AAA GCG GTT GCA TCG GCG GTC GTA GTC GTT GCG GCA GCG GGT AAC GAA GCG ACT TCG GCG AGC TCG
    TTT GCG GGT CAA CTA TTT CCG CAA CCG AGC AGC CCG CAG CAT CAG CAA CCG GGT CCG CCA TTG CTT CCG TCA AGC AGC TCG AGC
    Lys Ala Ala Val Asp Lys Ala Val Ala Ser Gly Val Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly Ser Ser
    140
    hinfI/aoyI
    shaII/bshI
    ofrI01
910 TCG ACA GTG GAC TAC CCG GCG AAA TAC CCG TCT TCT GTC ATT GCA GTA GCG GGT GTT GAC AGC AGC AAC CAA ACA GCA GCA TCT TTC
    AGC TGT CAC CTG ATG GCA CCG TTT ATG GCA AGA CAG TAA CCG CAT CCG CCA CAA CTG TCG TCG TCG GTT TCT GGT AGA AAG
    Ser Thr Val Asp Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala Val Gly Ala Val Asp Ser Ser Asn Glu Arg Ala Ser Phe
    170
    haeII
    hincII/hindII
    180
991 TCA AGC GTA GCA CCG GCT GAC GTT CAT GTC ATG GCA CCG GCG GTA TCT ATC CAA AGC AGC CTT CCG GCA AAC AAA TAC GCG GCG
    AGT TCG CAT CCG GCA CCG GAA CTA CAG TAC GCG GGT GCG CCG CAT ACA TAG GTT TCG TCG GAA GCA CCG TCG TTT ATG CCG GCG
    Ser Ser Val Gly Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr Leu Pro Gly Asn Lys Tyr Gly Ala
    190
    ppvNI
    accOI09I/draII
    bspI
    hgiCI
    kpnI
    hgiCI
    bspI
    asp718
    acc651
1072 TAC AAC GGT AGC TCA ATG GCA TCT CCG CAG CAG GTT GCG GCA GCG GGT GGT TCG ATT CTT TCT AGC CAG CCG AGC TCG ACA AAG
    ATG TTG CCA TGG AGT TAC CCG ACA GCG GCG CAA CCG CCG CCG CCG CCA GCA AAC TAA GAA ACA AAG TTC GTG GCG TTG ACC TGT TTG
    Tyr Asn Gly Thr Ser Met Ala Ser Pro His Val Ala Gly Ala Ala Leu Ile Leu Ser Lys His Pro Asn Trp Thr Asn
    220
    bglI
    bspBI
    230
1153 ACT CAA GTC GCG AGC AGT TTA GAA AAC AGC ACT ACA AAA CTT GGT CAT TCT TTC TAG TAT GCA AAA GCG CTG ATG AAC GTA
    TGA GTT CAG GCG TCG TCA AAT CTT TTG TCG TGA TGT TTT CAA CCA CTA ACA AAG ATA GCA CCG TTT CCG GAG TAG TTG CAT
    Thr Glu Val Arg Ser Ser Leu Glu Asn Thr Thr Thr Lys Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val
    250
    bolI/dan-I
    260
    270

```

Figure 6-2

8/17

WO 96/27671

1324 CAG GCG GCA GCT CAG TAA AACATAAA AACCGCCCTT CCGCCCGCCG GTTTTATT ATTTCCTC CTCCGCAATG TCAATCCCT
 CTC GCG GCT GCA CTC ATT TTGATTTT TTCCCGGCA CCGCGCGCCG CAAATAATA TAAATAACAG GAGCCGTACA AGTACGCCA
 Gln Ala Ala Gln Oc'

1321 GCATAATGCA CGGATGCTC CCGTCGAAA TTITAACAG AACCGCCGCG TTACCCCGG TCAATCCCT AACCGCCAG TCGTGAACG TCTCAATCCG
 GGTATTAGCT CCTACCCAG CGAGACTTTT AATTCCTC TTCCCGCCG AACTGCGCG AGTCAGGCA TTCCCGCTTC AGCACTTCG AGACTACCG

1321 CGCTTCCCGG TTCCCGCTCA GCTCAATCC GTACCGCTCG CCGCCCTTT CCGTATACCA GCACAGGCA TTCCGTATCG GATCGGAA TTGTACG
 CGAAGCGCC AAGCGCACT CGAGTACCG CATTCGCAAG CCGCCGAAA GAGTATGAG CCGTCGCGT AAGCAATAC CTAGCCCTT AACATTGCA

1321 TAAATTTTG TTAATATCG CGTTAAATTT TTGTTAATC AGCTCATTT TTAACCAATA CCGCCAAATC GCGAAATCG CTATTAATC AAAATAAG
 ATTATAAAC AATTATACG CCAATTAA AACATTAG TCGAGTAAA AATTCCTAT CCGCCTTAG CCGATTAG GAATATTAG TTTCCTATC

1321 ACCGAGTAG GGTGAGTGT TGTTCACAT TCGAACAGA GTCACTATT AAAGACGTG GACTGCAAG TCAAGCGCG AAAACCGTC TATCAGGCT
 TGGCTCTATC CCAACTACA ACAAGCTCA ACCTGCTCT CAGGTATATA TTCTTGAC CTACGCTCG ACTTCGCG TTTCGCAAG ATATGCCA

1321 ATGCGCCACT ACCGTACCA TCAACCTAAT CAGTTTTC CCGCTGCAAG TCGCGTAAAG CACTAAATCG GAACCTTAA CCGAGCGCCG GATTACAGC
 TACCGCGCTCA TCGACTTGT AGTCGATTA CTCAAAA CCGCACTCG ACAGCAATTC GTGATTAC CTTCGCAATT CCGTCGCGCG GTAAATCTCG

1321 TTGACGCGCA AAGCGCGCA ACGTCCGCA AAGCAAGCG AACCAACCA AACGACCGCG CCGTACGCG CTGCAAGTG TACCGGTAC GCTCGCGTA
 AACTGCGCCT TTCCGCGCT TCGACGCTC TTCTTCCG TTCTTCCCT TTCTGCGCG CCGATCGCG GACCTTCAG ATCCGCAATG CGAGCGCAT

1321 ACCAGCAAC CCGCGCGCT TAAATGCCG CTACAGCGCG CCGTCGATC KATCGCAAG CGAGCTGCA TCGCCTTCG CATTAATAT CTTCGCGTT
 TCGTGTGTG GCGCGCGCA ATACCGCG CATGTCCCG CGAGCGCTAG TGTAGGCTCG GCTCGCACT ACCGCAAGCG GTAAATATA GAAAGCGAA

Figure 6-3

[illegible]

Figure 6-4

Figure 6-5

11/17

Figure 6-6

12/17

4121 AACGCGAGC TAAAAAAGA ACGAAGTCGA GATGACGGAA TGACCTTATA AAATAAANA ACCAGCTGAA AAGCTGCTT TTTTGACG TTTGACCTT
 TTGCGCTCC ATTTTCTTCT TGCTTACGCT CTAGTCCCTT ACTCAATAT ATTAATTTT TCGTGACCT TTCACAGAA AAAAGTACG AAAGCTGAA
 4122 GTTCTTCTT ATUTGATAC ATATAGAAAT AACGTATTT TTATTTAGT TCGTGAAGG TCGCTTGAAG TGTGTATG TATGCTTTT AAAGTATTA
 CAACGAAGAA TAGAAGTATG TATATCTTTA TTGCAATAA ATATAATCA ACCAGCTTCC ACCCACTTC ACACGATAC ATACACAAA TTTCATACG
 4123 AAACCTTAA AATTGCTTC ACAGAAAAC CCGATCTGT AAAGTATAA GTGACTAAC AAATACATA ATAGATGCG GTTCTTTA ATATATATG
 TTTCGGAAT TTAACCAAG TGTCTTTG GGTAGACAA TTCAATAT CAGTATTTG TTATTTAT TATCAACC CAAGAAAT ATATATACG
 4124 TCGTAATAGT AGCATTTAT CAGATGAAA ATCAAGCTT TTAGTGACA ACAGAAAAG TCGAAAGTG AGACATGCA GAGAAAGA ATCGCTAT
 AGATATATCA TCGTAATAA GTTACTTTT TACTCCGA ATACAGCTT TGTCTTTC ACCTTCAC TCGTACCT CTCTTCTT TTAGCCTTA
 4125 GTTGATTAAT TGAAGTCTT GCATATCTT GAATTAANA AGCTGGAAG AGTAAGAT TGTGTCGAA TATTAGATA TAAACAAAT CCGTAACAG
 CAAGTATATCA AACTGACGA CGATATAGAA CTAAATTTT TCCAGCTTC TCAATTTCTA ACAGAGCTT ATATCTAT ATTTCTTTA GCACTTCTC
 4126 GCGAAGAAA GTGTATCGA GTGTGCTTT GTAAATCGAG GCTTGTGCA ATGTGCACT GAGAGAGAG ATGAAGAT GGCATTCAGT CACAAAGCT
 CGCTTCTT CAACATAGCT CAGACCAAA CATTAGCTC CGAAAGAGCT TACAGCTTGA CCGCTCTG TACTTTCTA CCGTAAGTCA GCTTTTCA
 4127 TGTGCTGAA GTTATTAAC AAAAGCAAC AGTTCGCTG TTGTTCTCA CATTACAGT TAAAACTT TATGATGCG AAGATTAATA TAAAGCTTG
 ACAAGCAGCT CAATATTTG TTTCGGTGG TCAAGCAAG ACAAGAGT GTATTTCTA ATTTTACA ATATACCC TTCTTAATTT ATTTCAAG
 4128 TCAAGATATG CTCAGGATT TCGCGCAATG ATGCAATATA AAAAATTTA TAAATCTT GTTGCTTTA TCGCTGCAAC GGAAGTACA ATAAATATA
 AGCTATACG GAGTTCCTAA AGCGCTTAC TACCTATAT TTTTAAT ATTTTACA CAACCAAAAT ACGCAGCTG CTTCACTGT TATTAATAT
 4129 AAGATAATTC TTATATACG CACATGCAATG TATTGCTATG TGTGACAGA ACTATTTTA AGAATACAGA AAAGTACGAT ATCAAAAG ATGATTTCA
 TTCTATTAAG ATATATATGTC GTGACGCTAC ATAAAGATAC ACAGCTTGT TCAATAAAT TCTATGCTT TTGATGCACT TTAGCTTTG TTAGCTAAGT

Figure 6-7

5321 ATTITGGAAA AAGCAATGCA AATTAGACTA TCAATCCAAAT GTAAACCTTC AATGATATCG ACCGAAATAAT AATATATAAT CGCATATACA ATCCGCAAT
TAAACCTTT TTCCTTACT TTAATCTGAT ACTAGCTTTA CATTTCCAG TTACTAGACC TCCCTTTTA TTATATTTA CGCATATATG TACCGCTTAA

5321 GACGAACTG CAAATATCG TGTAAAGAT ACCGATTTTA TACCGCATCA TGAAGAAAG AATTGAAAC GTTGTCTCA TTGTGACCA CCGTACAC
CTCCTTGAC GTTTATATAG ACATTTCCCA TCCCTAAAT ACTGCGTACT ACTCTTTT TTAACCTTC CAACAGACT AAGCTCTT CCAATATCG

5321 GTAAAGCTT AATCTCTAT CGTGGTTCT TAAAGAAAT ACATATAAA TTAACCTTC ATGACACAGA ACAAAGCAT TTGATTCATA CAGATATCA
CATTTCCAA TTACAGATA CCACGAACA ATTTCTTTA TGTATTTT AATTGGAAC TACTGTCT TCTTCCGCTA AACTAGATAT GTTACTACT

5321 CGAAAGCC GATCAAGATG GATTTCTAT TATTCGAATG TCGCATTCG AAGCGAAA TTATTTAT AAGAGTAT TCAAGAAAC CGCCACTTC
CGTTTCCG CTACTCTAC GTAAAGATA ATAAAGTAC ACCTTAAC TCCCTTTT AATAAATAA TTCTCATCA AGTGTCTTC CGCGTAAAC

5321 TTGAGATTA GATCTATTA TTCTATTTA AAGCATTCGA CGATGCTTAA GAGACAGT TATTAATAC TCAATAGAA CGGTGCTCT CAATATCT
ACCTCTAAT CTACGATAT ACAAATAT TTCTTACTT CCAACGATC GTTCTCTCA ATATATATG ACTATCTT CGCACAGAG GTTATATAGA

5321 TATTAGAA AGCAATCTA AATATATG AAGGCAAT GACAATAGT AATGACCA TATTAATGAC TACAGAGAA AGATGACA TTCTTACT
ATAATCTT TCGTTACAT TTAAATGAC TTTCCTTA CTCTATGAC TTACTGCT ATATATCT ATCTCTT TCTTACTT ACAGTACT

5321 AATTAGCA CGAATATTCG ATAAATATCG CGATGATCT AAGCGTATG GTTTATATG CTCTCTCT CGTACAGCT ATGCGGCTTA TTGCAATAT
TTAATCTT CTATATACC TATTAATAC CCACTAGAA TTCCGATTAAC GACAATAC GACAGAGCA GCACTGAC TACCGCGCAT AAGCTATTA

5321 GAGATGATGT GTGTATCT AACAAGCA CGACAGTCA GCCATGATG GACAACCGT GAGTGAAG TCGAAGTGA TTGTGATAC GACGAGATTC
CTCTACTACA CACAGTACG TTGTCTCT CGCTCAGT CGGATGAT CCACTCTTC ACCTTACTT AAGATATG CTCTCTGAG

5321 TACTAGATTA TGCATCTAG GTGAATGAG ATTCGCGCT TACAGATCT CAATTTCT CTATTTGC GATTATGAT TCAAGTGAAT ACTAGAGAA
ATGATCTAAT ACCTAGAGCT CACCTTACT TACCGCGCA ATGCTAGCA GTTAAAGA CATTAACCG GTAAATATCA AGTCAAGCTA TGAATCTCT

5321 TATTAGCA AGCAATCTA AATATATG AAGGCAAT GACAATAGT AATGACCA TATTAATGAC TACAGAGAA AGATGACA TTCTTACT
ATAATCTT TCGTTACAT TTAAATGAC TTTCCTTA CTCTATGAC TTACTGCT ATATATCT ATCTCTT TCTTACTT ACAGTACT

5321 AATTAGCA CGAATATTCG ATAAATATCG CGATGATCT AAGCGTATG GTTTATATG CTCTCTCT CGTACAGCT ATGCGGCTTA TTGCAATAT
TTAATCTT CTATATACC TATTAATAC CCACTAGAA TTCCGATTAAC GACAATAC GACAGAGCA GCACTGAC TACCGCGCAT AAGCTATTA

5321 GAGATGATGT GTGTATCT AACAAGCA CGACAGTCA GCCATGATG GACAACCGT GAGTGAAG TCGAAGTGA TTGTGATAC GACGAGATTC
CTCTACTACA CACAGTACG TTGTCTCT CGCTCAGT CGGATGAT CCACTCTTC ACCTTACTT AAGATATG CTCTCTGAG

5321 TACTAGATTA TGCATCTAG GTGAATGAG ATTCGCGCT TACAGATCT CAATTTCT CTATTTGC GATTATGAT TCAAGTGAAT ACTAGAGAA
ATGATCTAAT ACCTAGAGCT CACCTTACT TACCGCGCA ATGCTAGCA GTTAAAGA CATTAACCG GTAAATATCA AGTCAAGCTA TGAATCTCT

5321 TATTAGCA AGCAATCTA AATATATG AAGGCAAT GACAATAGT AATGACCA TATTAATGAC TACAGAGAA AGATGACA TTCTTACT
ATAATCTT TCGTTACAT TTAAATGAC TTTCCTTA CTCTATGAC TTACTGCT ATATATCT ATCTCTT TCTTACTT ACAGTACT

5321 AATTAGCA CGAATATTCG ATAAATATCG CGATGATCT AAGCGTATG GTTTATATG CTCTCTCT CGTACAGCT ATGCGGCTTA TTGCAATAT
TTAATCTT CTATATACC TATTAATAC CCACTAGAA TTCCGATTAAC GACAATAC GACAGAGCA GCACTGAC TACCGCGCAT AAGCTATTA

5321 GAGATGATGT GTGTATCT AACAAGCA CGACAGTCA GCCATGATG GACAACCGT GAGTGAAG TCGAAGTGA TTGTGATAC GACGAGATTC
CTCTACTACA CACAGTACG TTGTCTCT CGCTCAGT CGGATGAT CCACTCTTC ACCTTACTT AAGATATG CTCTCTGAG

5321 TACTAGATTA TGCATCTAG GTGAATGAG ATTCGCGCT TACAGATCT CAATTTCT CTATTTGC GATTATGAT TCAAGTGAAT ACTAGAGAA
ATGATCTAAT ACCTAGAGCT CACCTTACT TACCGCGCA ATGCTAGCA GTTAAAGA CATTAACCG GTAAATATCA AGTCAAGCTA TGAATCTCT

5321 TATTAGCA AGCAATCTA AATATATG AAGGCAAT GACAATAGT AATGACCA TATTAATGAC TACAGAGAA AGATGACA TTCTTACT
ATAATCTT TCGTTACAT TTAAATGAC TTTCCTTA CTCTATGAC TTACTGCT ATATATCT ATCTCTT TCTTACTT ACAGTACT

5321 AATTAGCA CGAATATTCG ATAAATATCG CGATGATCT AAGCGTATG GTTTATATG CTCTCTCT CGTACAGCT ATGCGGCTTA TTGCAATAT
TTAATCTT CTATATACC TATTAATAC CCACTAGAA TTCCGATTAAC GACAATAC GACAGAGCA GCACTGAC TACCGCGCAT AAGCTATTA

5321 GAGATGATGT GTGTATCT AACAAGCA CGACAGTCA GCCATGATG GACAACCGT GAGTGAAG TCGAAGTGA TTGTGATAC GACGAGATTC
CTCTACTACA CACAGTACG TTGTCTCT CGCTCAGT CGGATGAT CCACTCTTC ACCTTACTT AAGATATG CTCTCTGAG

5321 TACTAGATTA TGCATCTAG GTGAATGAG ATTCGCGCT TACAGATCT CAATTTCT CTATTTGC GATTATGAT TCAAGTGAAT ACTAGAGAA
ATGATCTAAT ACCTAGAGCT CACCTTACT TACCGCGCA ATGCTAGCA GTTAAAGA CATTAACCG GTAAATATCA AGTCAAGCTA TGAATCTCT

5321 TATTAGCA AGCAATCTA AATATATG AAGGCAAT GACAATAGT AATGACCA TATTAATGAC TACAGAGAA AGATGACA TTCTTACT
ATAATCTT TCGTTACAT TTAAATGAC TTTCCTTA CTCTATGAC TTACTGCT ATATATCT ATCTCTT TCTTACTT ACAGTACT

5321 AATTAGCA CGAATATTCG ATAAATATCG CGATGATCT AAGCGTATG GTTTATATG CTCTCTCT CGTACAGCT ATGCGGCTTA TTGCAATAT
TTAATCTT CTATATACC TATTAATAC CCACTAGAA TTCCGATTAAC GACAATAC GACAGAGCA GCACTGAC TACCGCGCAT AAGCTATTA

5321 GAGATGATGT GTGTATCT AACAAGCA CGACAGTCA GCCATGATG GACAACCGT GAGTGAAG TCGAAGTGA TTGTGATAC GACGAGATTC
CTCTACTACA CACAGTACG TTGTCTCT CGCTCAGT CGGATGAT CCACTCTTC ACCTTACTT AAGATATG CTCTCTGAG

5321 TACTAGATTA TGCATCTAG GTGAATGAG ATTCGCGCT TACAGATCT CAATTTCT CTATTTGC GATTATGAT TCAAGTGAAT ACTAGAGAA
ATGATCTAAT ACCTAGAGCT CACCTTACT TACCGCGCA ATGCTAGCA GTTAAAGA CATTAACCG GTAAATATCA AGTCAAGCTA TGAATCTCT

5321 TATTAGCA AGCAATCTA AATATATG AAGGCAAT GACAATAGT AATGACCA TATTAATGAC TACAGAGAA AGATGACA TTCTTACT
ATAATCTT TCGTTACAT TTAAATGAC TTTCCTTA CTCTATGAC TTACTGCT ATATATCT ATCTCTT TCTTACTT ACAGTACT

5321 AATTAGCA CGAATATTCG ATAAATATCG CGATGATCT AAGCGTATG GTTTATATG CTCTCTCT CGTACAGCT ATGCGGCTTA TTGCAATAT
TTAATCTT CTATATACC TATTAATAC CCACTAGAA TTCCGATTAAC GACAATAC GACAGAGCA GCACTGAC TACCGCGCAT AAGCTATTA

5321 GAGATGATGT GTGTATCT AACAAGCA CGACAGTCA GCCATGATG GACAACCGT GAGTGAAG TCGAAGTGA TTGTGATAC GACGAGATTC
CTCTACTACA CACAGTACG TTGTCTCT CGCTCAGT CGGATGAT CCACTCTTC ACCTTACTT AAGATATG CTCTCTGAG

5321 TACTAGATTA TGCATCTAG GTGAATGAG ATTCGCGCT TACAGATCT CAATTTCT CTATTTGC GATTATGAT TCAAGTGAAT ACTAGAGAA
ATGATCTAAT ACCTAGAGCT CACCTTACT TACCGCGCA ATGCTAGCA GTTAAAGA CATTAACCG GTAAATATCA AGTCAAGCTA TGAATCTCT

5321 TATTAGCA AGCAATCTA AATATATG AAGGCAAT GACAATAGT AATGACCA TATTAATGAC TACAGAGAA AGATGACA TTCTTACT
ATAATCTT TCGTTACAT TTAAATGAC TTTCCTTA CTCTATGAC TTACTGCT ATATATCT ATCTCTT TCTTACTT ACAGTACT

5321 AATTAGCA CGAATATTCG ATAAATATCG CGATGATCT AAGCGTATG GTTTATATG CTCTCTCT CGTACAGCT ATGCGGCTTA TTGCAATAT
TTAATCTT CTATATACC TATTAATAC CCACTAGAA TTCCGATTAAC GACAATAC GACAGAGCA GCACTGAC TACCGCGCAT AAGCTATTA

5321 GAGATGATGT GTGTATCT AACAAGCA CGACAGTCA GCCATGATG GACAACCGT GAGTGAAG TCGAAGTGA TTGTGATAC GACGAGATTC
CTCTACTACA CACAGTACG TTGTCTCT CGCTCAGT CGGATGAT CCACTCTTC ACCTTACTT AAGATATG CTCTCTGAG

5321 TACTAGATTA TGCATCTAG GTGAATGAG ATTCGCGCT TACAGATCT CAATTTCT CTATTTGC GATTATGAT TCAAGTGAAT ACTAGAGAA
ATGATCTAAT ACCTAGAGCT CACCTTACT TACCGCGCA ATGCTAGCA GTTAAAGA CATTAACCG GTAAATATCA AGTCAAGCTA TGAATCTCT

5321 TATTAGCA AGCAATCTA AATATATG AAGGCAAT GACAATAGT AATGACCA TATTAATGAC TACAGAGAA AGATGACA TTCTTACT
ATAATCTT TCGTTACAT TTAAATGAC TTTCCTTA CTCTATGAC TTACTGCT ATATATCT ATCTCTT TCTTACTT ACAGTACT

5321 AATTAGCA CGAATATTCG ATAAATATCG CGATGATCT AAGCGTATG GTTTATATG CTCTCTCT CGTACAGCT ATGCGGCTTA TTGCAATAT
TTAATCTT CTATATACC TATTAATAC CCACTAGAA TTCCGATTAAC GACAATAC GACAGAGCA GCACTGAC TACCGCGCAT AAGCTATTA

5321 GAGATGATGT GTGTATCT AACAAGCA CGACAGTCA GCCATGATG GACAACCGT GAGTGAAG TCGAAGTGA TTGTGATAC GACGAGATTC
CTCTACTACA CACAGTACG TTGTCTCT CGCTCAGT CGGATGAT CCACTCTTC ACCTTACTT AAGATATG CTCTCTGAG

5321 TACTAGATTA TGCATCTAG GTGAATGAG ATTCGCGCT TACAGATCT CAATTTCT CTATTTGC GATTATGAT TCAAGTGAAT ACTAGAGAA
ATGATCTAAT ACCTAGAGCT CACCTTACT TACCGCGCA ATGCTAGCA GTTAAAGA CATTAACCG GTAAATATCA AGTCAAGCTA TGAATCTCT

5321 TATTAGCA AGCAATCTA AATATATG AAGGCAAT GACAATAGT AATGACCA TATTAATGAC TACAGAGAA AGATGACA TTCTTACT
ATAATCTT TCGTTACAT TTAAATGAC TTTCCTTA CTCTATGAC TTACTGCT ATATATCT ATCTCTT TCTTACTT ACAGTACT

5321 AATTAGCA CGAATATTCG ATAAATATCG

Figure 6-8

6221 ACTGTATCAA ACTGCTAAT CGGTAGAAC GCAACGCTC CAGCATGCCA TTGTGCGCT TATCGTAGAA GACCTGTTG AATATGCAGG CAATGGCGT
 TCACATAGCT TCAACGATTTA GGCATCTTCC GGTTCGAA GTCCTACGCT AATACGCGCA ATAGCATCTT CTCGACAAAC TTATACGCTC GTTACCGCA

bsp14061
 bsp1386
 bsp1
 bsp1/ksp6321

6321 AATATTCGTG TCGAAGGAGC GACACATTT CTACCATCTT TCACTGTACA CGTAGCAATG GCAGGTGCCA TGTGATTCG TCTGCATCAT CCGATCTGTT
 TTATAACGAC AGCTTCCTCG CTGTTGTAAA GATGGTAGCA ACTGACATCT CCACTGCTTAC GCTGCACGGT AGACTAACCC ACACGTACTA GCGTAGACAA

ssp1
 bsp14071
 bsp1
 bsp1
 hgl1
 bsp1
 hlnoi/hind11

6421 ATACGACGAG CGCTTCGCTC TTAAGTGAAG CAGTTAAGCA ATCAGATCTT CCTTCACGTT ATGACCATCT GTCCGAGTTC GTAATGTCTG GTCACTTTC
 TATGCTGCTC GCGAAGCCAG AATTGACTTC GTCAATTCCT TAGCTAGAA GGAAGTCGAA TACTGCTACA CACGCTCAAG CATTACAGAG CAGTTCAAG

hae1
 eco4711
 eco571
 bgl1
 eco571

6521 CGACTGTGAG AACTCTCTCG AATCGCTAGA GAAATTCCTG AATCGCATTC AGAGGTGAC AGAAGGACAC GCATATATAG TCGATCTGTC AAACGCGTA
 GCTGACATCT TTGAAAGACC TTAGCGATCT CTAAACACC TTACCTTAG TCTTCACCTG TCTTCCTCTG CGTATATATC ACCTACACAG TTTCGCTAT

apol
 ssp1
 bsp1
 bsp1

6621 GCATTTCGAA CGATGACCTC TAATAATTCT TAATCATGCT GCTTACGAT TTATTACTT CTCCTAGTAT TAGATATTAT CATGGCTGTC ATGGCGATT
 GGTAAACTT GCTACGTGAG ATTATTACAA ATTACTACAA CCAATGCATA AATAATTGAA GAGCATGATA ATCATTAATA GTACCGACAG TACCGCGTAA

ssp1
 bsp1
 bsp1
 bsp1

6721 AACGGAATGA AGGCTGCTCT TAATCGCGCG CATTTCGCT AATAAGAAA AGCATTAAT ATGACCGAAT TCAATTATA ATAGCGTAAT AGATTACAT
 TTGCTTATTT TCCGACAGCA ATTACGCGCG GTAAACGCA TTATTCCTTT TCGTAAATTA TACGCGCTTA ACTTAATTAT TATTCATTTA TCTAATGTA

ssp1/asp1/vsp1
 ssp1/asp1/vsp1

6821 TAGAATAATGA AAGCGATTT TATCGCTGAG AATGTTACAG TCTATCCCGG CAAATGTTAC CCFATTAATC AGATPAAGAA AGAAGGAT TTTCGCTAC
 ATCTTTACT TTCGCTTAA ATAGGACCTC TTACATGTC AGATAGGCGC GTTATCAATG GCAATATATG TCTATTCCT TCTTTCCTA AAACGCGAT

sheli/drai
 sheli/drai

6921 GCTCAATGTC TTAAAAAA CACAAAGAC CACATTTTT AATGCTGCT TTATTCCTCA ACTAAGGAC CCAATGCTC AACAAAGAA AATTGATTA
 CGACTTAGG AATTTTTT GTGTTTCTG GTGTAAAA TTACACGAGA AATAGAGAT TCAATTCTG GGTATCAAG TTGTTGCTT TTAACCTAT

sheli/drai
 sheli/drai

7021 AGTGGGATAT TTAAAAATA TATATTATG TTACAGTAAT ATTACCTTT AAAAAAGAT TCAATCTAAT GAAAGAGCA GACAGTAG CCTCGTAAT
 TCAACCTATA AAAAAATTA ATATAATAC AATGCTATTA TAACGAAA TTTTTCCTA ACTAAGATTA CTTCCTTCCT CTGTTCAATC GAGCATTTA

ssp1
 sheli/drai
 ssp1
 ssp1

7121 TCACTTAGA TAAAAATTTA GAGGCGATAT CAATGACAT TTAATTAAT TCAATTAGAC AATTGAGAG CAAACGAG ATTAATCAT TATTGAAAC
 AGTGAATCT ATTTAAAT CCTCGTATA GTTACTTCA AATTAATTA ACTAATCTG TTAACCTCT CTTCCTCTA TAAATAGTA ATAACTTGG

apol
 ssp1
 ssp1
 ssp1

Figure 6-9

7221 AACAAAGCAG TTTTAGTATA ACCAGACAAA TTGATATTAG TGTTTATAC CGAAGCATAA AACAGAGC ATATAAATT TACCGTCAG TTAATTTCTT
TCTTTGCTG AAATCATAT TCGTGTCTTT AACTATATC ACAAAATATG GCTTGATAT TCTCTTCC TATATTAA ATCGACGTA AATAAAGAA
7231 AGTGACAAG GTGATAACT CAATACAGC TTTTAGACT GGTACATA CGCAGCGACA GTTAGCTAT TCGGATAGT TAGAGCCAGT TTAACAATT
TCAGCTTCC CACTATTGA GTTATGTCC AAATGTGA CGAATGTAT GCGTCCCTT CAATCGAATA ACCGATTA ATCTCGCTA AATATCTTAA
7421 TTGATGCTG TATCTAAG ATTCTGTGT ATTGCACTC CTGTAAAGAA TGACTTCAA GAGTTTATG ATTATAGT TTCTCATGA GACAAATATA
AAACTAGCAC ATAGATTTG TAGAGACGA TAAAGCTGAG GACATTCTT ACTAGATT CTGAAATAG TAAATATGA AAGCTAGAT GTCTTAATAT
7521 ATGCTGCGG GAAATGTTT CGCAAGAC CTATAGCTGA AAATGCTTT TCTTTCTTA TTAATCGATG GACTGATT ACTGCGTTA ACTTAATAT
TACCAAGCCG CTTAAGAA GCGTTTGTG GATATGAGT TTAACGAAA ACAGAAAGAT AATAAGTAG CTGAGTAA TGACCGAAT TCAATTATTA
7621 CAATATATAT AGTAATTAC TTCTAGCCAT TATTACAGA CGAAATTTCA TTAATTAAG TAAATCAATA TATTACCGG TATCTTACA GGTATCAT
GTATATTAT TCAATTAATG AAGATGGTA ATATGTCTT CCTTTAAGT AATTAATTC ATTAAGTAT ATAAATCGG ATAGAAATGT CCAATAGTA
7721 TCTGTTTGT ATGCTTATCA TCGAGCATTG TTAATCAAT CTATTCAGCA ATTCTCAGAT AGCGCTAATG ACTGCTTTT ATAAATATAG ATAAAGCGCA
AGACAAAGAC TACCAATAGT AGCTCTAAC AAATACTTGA GATAAGTCTT TAACAGCTTA TCGGATTAAG TGACCGAAA TATTATATCT TATTACGCT
7821 CTGTAATTT TACAGTCGCT TTTCTAATGT CACTAAGCTG CCGCGTATG TGAAGAGAGT TTTATATTA CAGCTACAGA TCGATATGCT TCTTTCTG
GACATGAAA ATGTCAGCCA AAAGATTACA GTCAATTGAG GCGCGAATCA ACTTTCTCA AAAATATAT GTCAAGCTT AGCTATAGCA ACATAAGAC
7921 AACGACTTC TCGTTTTCG CTCTTATAT CGAATGCTT TATGACGCT GAGCTGCGA AGCGATATAG TGTCTTATC TTAATCTGA ACTGCTGCG
TTGCTGTAG AGCAAAAGC GAAATAATTA GTTAAAGCA ATAACTGCA CTGCGAGCT TCGATATAT ACACATATG AATATAGCT TACCAAGCG
8021 GGAAGAGCG AAAATGCTG ACATTGCTC CAGCTAATA GAGAGGATTT ACATATAGT TATGAGCTT GTACATGCA AAAAGTGAAA TGAGATCT
CCTTCTGCT TTTAAGGAG TGTAAACAG GTGCAATTTT CCTGCGTAA TGTATACTCA ATAGCGAAA CATCTAGT TTTCACTTT ACTGCTAGT

Figure 6-10

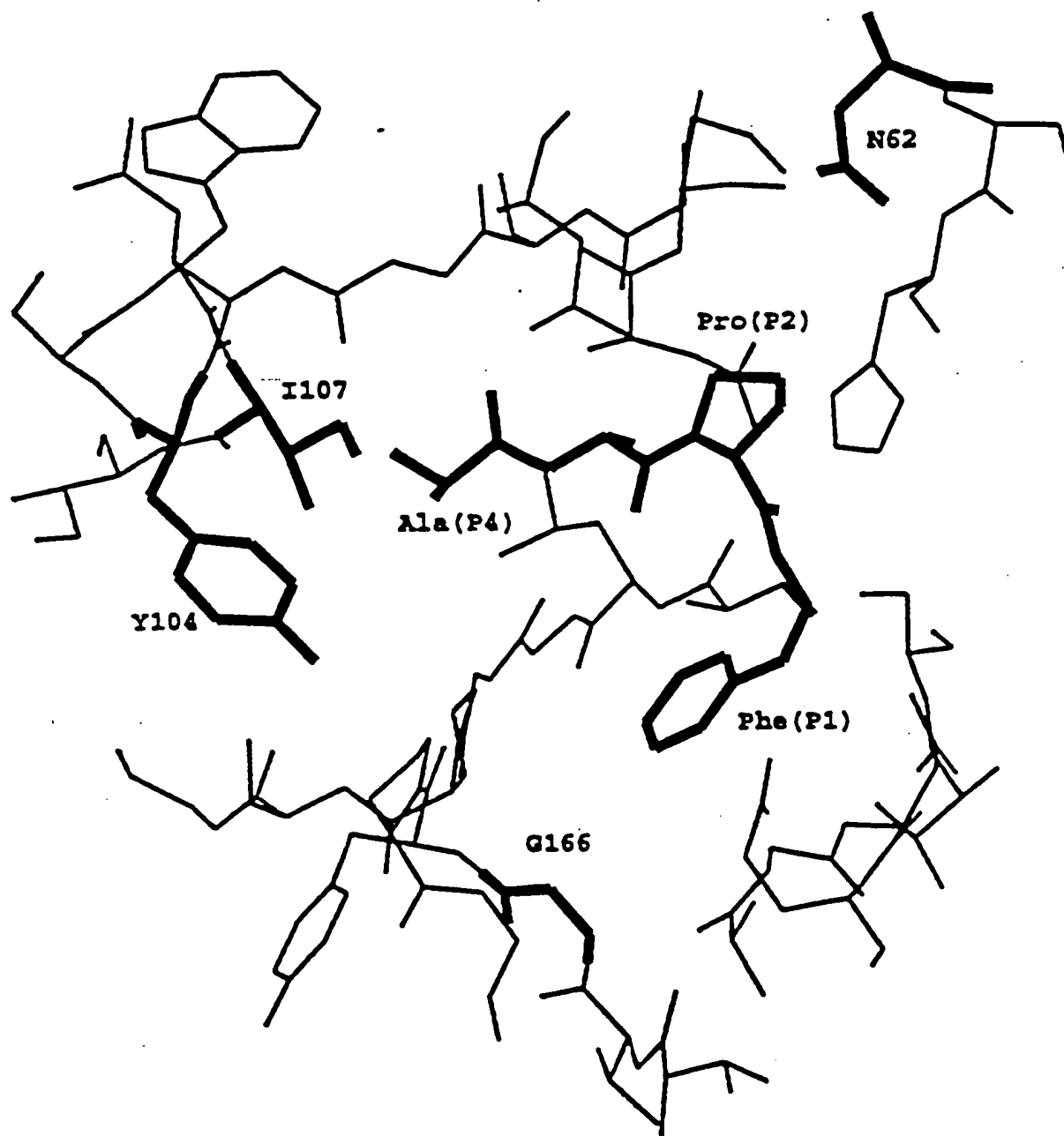


FIGURE 7

INTERNATIONAL SEARCH REPORT

International Application No.

PL./US 96/02861

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/57 C12N9/54 C12N1/21 C12P21/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROTEIN ENGINEERING, vol. 4, no. 7, 1991, pages 719-737, XP002008733 R. SIEZEN ET AL: "Homology modelling and protein engineering strategy of subtilases, the family of subtilisin-like serine proteinases"	1-23, 25-27, 29
Y	see the whole document especially figure 3, table V and page 729, right column, last paragraph-page 730 and Discussion --- -/--	1-29

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"A" document member of the same patent family

Date of the actual completion of the international search

18 July 1996

Date of mailing of the international search report

31.07.96

Name and mailing address of the ISA

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 Fax (+31-70) 340-3016

Authorized officer

Van der Schaal, C

INTERNATIONAL SEARCH REPORT

International Application No

PL./US 96/02861

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 84, August 1987, WASHINGTON US, pages 5167-5171, XP002008734 J. WELLS ET AL: "Recruitment of substrate-specificity properties from one enzyme into a related one by protein engineering" see the whole document ---	1-29
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 84, March 1987, WASHINGTON US, pages 1219-1223, XP002008735 J. WELLS ET AL: "Designing substrate specificity by protein engineering of electrostatic interactions" see the whole document ---	2-6, 11-13
A	SCIENCE, vol. 233, 1986, pages 659-633, XP002008736 D. ESTELL ET AL: "Probing steric and hydrophobic effects on enzyme-substrate interactions by protein engineering" see the whole document ---	2-6, 11-13
A	ANNALS NEW YORK ACADEMY OF SCIENCES, vol. 672, 1992, pages 71-79, XP002008737 T. GRAYCAR ET AL: "Altering the proteolytic activity of subtilisin through protein engineering" see the whole document ---	2-6
Y	BIOCHEMISTRY 32 (5). 1993. 1199-1203. CODEN: BICHAW ISSN: 0006-2960, XP002008738 RHEINNECKER M ET AL: "ENGINEERING A NOVEL SPECIFICITY IN SUBTILISIN BPN." see the whole document ---	2-6
A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 267, no. 23, 15 August 1992, MD US, pages 16335-16340, XP002008739 K. NAKAYAMA ET AL: "Consensus sequence for precursor processing at mono-arginyl sites" see the whole document ---	1,7,24, 25,28,29
Y	EP,A,0 316 748 (HOECHST AG) 24 May 1989 see the whole document ---	24,28
Y	WO,A,91 11454 (UPJOHN CO) 8 August 1991 see the whole document ---	24,28
-/--		

INTERNATIONAL SEARCH REPORT

International Application No
PC./US 96/02861

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,95 30010 (PROCTER & GAMBLE) 9 November 1995 see claim 1	1-23,26, 27
X	EP,A,0 405 901-(UNILEVER PLC ;UNILEVER NV (NL)) 2 January 1991 see claim 1	1-23,26, 27
P,X	BIOCHEMISTRY 34 (41). 1995. 13312-13319. ISSN: 0006-2960, 17 October 1995, XP002008740 BALLINGER M D ET AL: "Designing subtilisin BPN' to cleave substrates containing dibasic residues." see the whole document	7-13, 18-24, 28,29
Y		1-6, 14-17, 25-27
P,Y	KEYSTONE SYMPOSIUM ON PROCESSING ENZYMES: BIOCHEMISTRY, GENETICS AND CLINICAL RELEVANCE, LAKE TAHOE, CALIFORNIA, USA, MARCH 2-8, 1995. JOURNAL OF CELLULAR BIOCHEMISTRY SUPPLEMENT 0 (19B). 1995. 233. ISSN: 0733-1959, 4 March 1995, XP002008741 WELLS J A ET AL: "Subtilisin: An enzyme made for engineering." see abstract B7-012	1-29